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**AN IMPROVED *IN VITRO* MODEL FOR THE STUDY OF  
*MYCOBACTERIUM LEPRAE*/SCHWANN CELL INTERACTIONS**

A Dissertation

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
in partial fulfillment of the  
requirements for the degree of  
Doctor of Philosophy

in

The Department of Biological Sciences

by  
Deanna Alisa Hagge  
B.S., Louisiana State University, 1996  
December 2001

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## ABSTRACT

Globally, millions of leprosy patients suffer irreversible peripheral nerve damage resulting in blindness or other disabilities as a consequence of *Mycobacterium leprae* infection. Schwann cells, the neural target of *M. leprae*, have a central role in leprosy histopathology including axonotrophy, altered myelin architecture and demyelination. The mechanisms of nerve damage have not been fully elucidated but appear to be the direct result of *M. leprae* within Schwann cells or a combined effect with an aggressive immune response to *M. leprae* within the nerves. There is no standardized *in vitro* model for the study of *M. leprae* interactions with the Schwann cell that preserves the viability of *M. leprae*. Recent studies have determined that 33<sup>0</sup> C is permissive for maintenance of short-term *M. leprae* viability in axenic medium. Using this information, we developed a Schwann cell infection model that preserves the metabolic activity of *M. leprae* for up to 3 weeks in cultures, maintains functional abilities of Schwann cells to interact with neurons in culture and mimics cooler temperature areas where infection is observed in patients. When this model was used to study the impact of *M. leprae* infection on Schwann cells, Schwann cells had altered interactions with other Schwann cells and altered gene expression encoding several important adhesion molecules such as neural cell adhesion molecule (NCAM), neural-cadherin (N-cadherin) and glial fibrillary acidic protein (GFAP). However, *M. leprae*-infected Schwann cells were able to align and associate with neurons in culture, proliferate along these neurons and subsequently produce myelin in a manner comparable to control cultures. Established myelinated Schwann cell/neuron co-cultures, maintained at 33<sup>0</sup> C, did not demonstrate any apparent alterations in Schwann cell/neuron interactions nor myelin architecture due to *M. leprae* infection. These results indicate that *M. leprae* infection at the multiplicity of infection used for these studies does not appear to have

detrimental effects on Schwann cell functional capabilities in the peripheral nerve. Therefore, the neuropathy observed in leprosy is most likely due to an aggressive immune response to infection within the nerve and not direct effects of *M. leprae* upon the Schwann cell.



## **CHAPTER 1. INTRODUCTION**

### **1.1. Discovery of Etiologic Agent, Epidemiology and Treatment**

*Mycobacterium leprae* was first observed in 1874 in Bergen, Norway by Dr. G. Armauer Hansen in unstained fluid collected from the skin lesions of leprosy patients (Meyers, W.M. and Marty, A.M., 1991). This bacterium was accepted as the causative agent of leprosy, and diagnosis of the disease was based upon clinical and histopathological findings of the bacteria within biopsies or skin scrapings taken from the patient.

There are currently more than 600,000 leprosy cases registered for treatment worldwide with more than 690,000 new cases diagnosed annually (World Health Organization, 2000). More than one-fourth of all reported leprosy patients worldwide have disease-related disabilities and approximately 50% of those are severely disabled (World Health Organization, 1995). Leprosy is the leading cause of non-traumatic nerve damage and the third leading cause of blindness due to an infectious agent (Johnson, P.C., 1997; Gillis, T.P. and Hastings, R.C., 1992).

An effective multi-drug therapy (MDT) is available consisting of dapsone (100 mg daily), clofazimine (10-14 mg daily) and rifampin (600 mg monthly) that can render a patient free of live *M. leprae* within six months of treatment. The MDT strategy implemented by the World Health Organization nearly two decades ago has effectively reduced the global prevalence of the disease by 85%; however, the incidence of leprosy still remains near 1.4 per 10,000 on a global basis with most of the cases concentrated in India, Brazil, Indonesia and Bangladesh (Fox, J.L., 2001). This drug-based eradication strategy is projected to fall short of the goal of leprosy elimination (< 1 per 10,000) because the development of drug resistance is considered inevitable (Fox, J.L, 2001).

Irreversible nerve damage has also been shown to continue to develop during and even after antimicrobial therapy (Gillis, T.P and Hasting, R.C., 1992; Rambukkana, A., 2000). It is estimated that therapeutic intervention has prevented only one-third of infected individuals from suffering further nerve damage. There are 2-3 million persons that are physically disabled due to past or present *M. leprae* infections (WHO Bulletin, 1998). Due to the higher prevalence of leprosy in poorer third-world populations, treatment and diagnosis within those regions can be limited and the disability of patients can result in a substantial economic burden for the affected individual, family and nation.

Transmission of the disease is thought to be person to person through intact skin, inhalation and deposition of bacilli onto intact nasal mucosa or penetration wounds such as those made by thorns or biting arthropods (Hastings, R.C. *et al*, 1988). Most individuals exposed to the disease effectively resist infection even in highly endemic areas. It is estimated that for every 200 individuals infected, only one will develop an overt case of the disease and an incubation period of 2-4 years post infection is average for disease to become noticeable by the patient who then may seek medical assistance.

## **1.2. Clinical Manifestations of Disease**

Clinical evidence of disease is usually characterized by nerve paralysis, anaesthesia, tingling, numbness and burning sensations, thickening of nerves, and occasional weakness (Chimelli, L. *et al*, 1997). Diagnosis is based upon finding evidence of acid-fast bacilli in the skin or nerve. In several laboratories, polymerase chain reaction (PCR) is currently used to complement histopathological and clinical analysis of patient biopsies (Williams, D.L. *et al*, 1990; Scollard, D.M., 2000).

Leprosy presents a spectrum of host response to *Mycobacterium leprae* infection. This can range from no disease, indeterminant disease which quickly resolves, a localized, granulomatous disease (tuberculoid) with very few demonstrable acid-fast bacilli to a widespread, progressive, anergic disease (lepromatous) with enormous numbers of bacilli (Hastings, R.C. *et al*, 1988; Gillis, T.P. and Krahenbuhl, J.L., 1998). Clinical manifestations are currently thought to be a result of the host's immune response to *M. leprae* infection, though the influence of *M. leprae* strain differences has not been ruled out. The tuberculoid (TT) form of the disease is typified by a cellular immune response to infection with few demonstrable bacteria, one or few circumscribed lesions and well formed granulomas with central accumulations of epithelioid and occasional Langerhans' giant cells. When the host's immune system responds with antibody or humoral immunity instead of cell-mediated immunity, the lepromatous (LL), or disseminated, form of infection develops. The antibody response is much less effective at limiting bacilli growth and spread; therefore, *M. leprae* populations can reach  $10^{10}$  to  $10^{11}$  bacilli per gram of tissue.

Between the two polar forms of tuberculoid and lepromatous leprosy, there also exists a spectrum of borderline clinical manifestations that range from borderline tuberculoid (BT) to mid-borderline (BB) to borderline lepromatous (BL). These borderline forms represent dynamic stages of the disease that typically are unstable as they tend to upgrade or downgrade toward one of the polar forms as the host's immunity either develops a cellular or humoral immune response to infection (Adams, L.B. and Krahenbuhl, J.L., 1996).

Involvement of the nerves in the early stages of lepromatous leprosy is much less severe than that seen in tuberculoid leprosy due to the lack of cell-mediated immunity directed at the infected area. Deformities and damage may occur as the disease progresses (Gillis, T.P. and

Hastings, R.C., 1992). Since damage to the nerve is thought to occur as a result of very large numbers of *M. leprae* (lepromatous) or an aggressive cellular immune response to few *M. leprae* (tuberculoid), the reversal of the host's immune response, referred to as reversal reactions, from the lepromatous polar form to the tuberculoid form can cause dramatic damage and pain to infected areas (Gillis, T.P. and Hastings, R.C., 1992; Adams, L.B. and Krahenbuhl, J.L., 1996).

### **1.3. The Bacillus**

#### **1.3.1. Description**

*M. leprae* has the longest known generation time (11-13 days) of all known pathogenic bacteria for humans (Fujiwara, T., 1986). Genomic analysis has placed *M. leprae* within the order of *Actinomycetales* (Meyers, W.M. and Marty, A.M., 1991). This bacillus is 0.3-0.5 x 4.0-7.0  $\mu\text{m}$  in size and has mycolic acids present in the cell wall that are thought to be responsible for the acid-fast staining characteristic of the bacteria.

#### **1.3.2. Growth**

*M. leprae* is an obligate intracellular parasite and is typically found residing within endothelial, perineural, Schwann cells and macrophages in the cooler portions of the body such as the skin, anterior portion of the eyes, upper respiratory tract, peripheral nerves in the extremities or near the surface of the skin and the testes (Meyers, W.M. and Marty, A.M., 1991). The bacteria are uncultivable on axenic medium (Hastings, R.C. *et al*, 1988; Adams, L.B. *et al*, 2000; Truman, R.W. and Krahenbuhl, J.L., 2001). Even when maintained in the best axenic medium currently available (7H12), the bacteria have been shown to consistently lose 90% of their original metabolic activity within three weeks of removal from host tissue (Truman, R.W. and Krahenbuhl, J.L., 2001).

*M. leprae* is currently propagated and obtained for experimental purposes within athymic BALBc nude mouse footpad or the nine-banded armadillo (*Dasypus novemcinctus*). The armadillo develops a systemic infection which can yield  $10^{12}$  bacilli per animal within three years of inoculation (Truman, R.W. and Sanchez, R., 1993). The inoculated nude mouse foot pad develops an enormous foreign-body type macrophage granuloma loaded with up to  $10^{10}$  bacilli within 6 months (Chehl, S. *et al*, 1983; Adams, L.A. and Krahenbuhl, J.L., 1996). Although the mouse yields less per animal, the bacteria obtained are of higher viability and are therefore more desirable for *in vitro* studies purporting to model live *M. leprae* infection (Truman, R.W. and Krahenbuhl, J.L., 2001).

### **1.3.3. Viability Assessment**

Viability analysis in the past was limited to 6-12 month growth assays using appropriate animal hosts (Franzblau, S.G., 1989). In the last few decades, however, the Buddemeyer and BACTEC 460 radiorespirometric assay systems, which provide dependable results within one to two weeks, have become the gold standard for measuring the metabolic activity of *M. leprae in vitro* (Franzblau, S.G., 1989; Truman, R.W. and Krahenbuhl, J.L., 2001). Both the Buddemeyer and the BACTEC 460 systems measure the ability of *M. leprae* in axenic medium to oxidize  $C^{14}$  radiolabelled palmitic acid to radiolabelled carbon dioxide which is then quantitated by a scintillation counter (Ganantra, R.D. *et al*, 1980; Franzblau, S.G., 1989). *M. leprae* is maintained for a short period within this system, but does not replicate (Franzblau, S.G., 1989). These assays have been shown to directly correlate with viability measurements using the mouse footpad *in vivo* system (Barclay, R. and Wheeler, P.R., 1989; Hastings, R.C. and Franzblau, S.G., 1988; Hastings, R.C. *et al*, 1988).

Even though the BACTEC 460 system is somewhat simpler to perform, it requires approximately ten times as much bacterial inoculum as that of the Buddemeyer assay and is 2-4 times as expensive to perform. Within the Buddemeyer system,  $10^7$  *M. leprae* usually will yield 2-10,000 counts per minute (CPM) of CO<sub>2</sub> per day compared to a growth index (GI) of 21-69 when incubated within the BACTEC system (Franzblau, S.G., 1989). Therefore, the BACTEC system cannot be used to assess viability or drug susceptibility directly from patient specimens unless heavily infected; however, it has been used for experimental drug screening and bacterial harvest viability measurement (Franzblau, S.G., 1989; Truman, R.W. and Krahenbuhl, J.L., 2001),

#### **1.3.4. Animal Disease Models**

Many different animal species have been analyzed to find a suitable model for leprosy research. However, no ideal animal model for leprosy has been found which mimics the human disease-state. In 1959, Dr. Charles Shepard discovered that the mouse footpad supported the multiplication of *M. leprae* (Shepard, C.C., 1960); however, nerve damage within this model is relatively minor and slow to develop (de Blaquiére, G.E. *et al*, 1994). Therefore, this animal does not provide a good model of disease.

In 1968, Dr. Waldemar Kirchheimer and Dr. Eleanor Storrs discovered that the nine-banded armadillo was highly susceptible to the development of a disseminated form of *M. leprae* infection (Kirschheimer, W.F. and Storrs, E.E., 1971). The armadillo has become a well-recognized model of lepromatous disease which has a similar form of nerve involvement in distribution and histopathology to the human disease state (Scollard, D.M., 2000). However, the armadillo is not a convenient laboratory animal because it does not breed in captivity or care for its young. Therefore, all armadillos must be wild caught, quarantined and held in specialized

stalls for experimental infection studies. This is very cost prohibitive. In addition, there are no armadillo-specific reagents necessary for immunological, protein or genomic analyses.

Neonatally thymectomized or congenitally athymic mice, which lack the ability to mount a T lymphocyte immune response to infection, have been shown to develop a disseminated *M. leprae* infection. The infected footpad tissue of these animals develops into an enormous foreign body-type macrophage granuloma within 6-9 months that has become the 'gold standard' for the harvest of fresh, highly viable bacteria (Chehl, S. *et al*, 1983; Truman, R.W. and Krahenbuhl, J.L., 2001). The lack of an intact immune system, though, makes this model also inappropriate for the study of nerve damage within a presumably immunocompetent host.

Primates such as monkeys and chimpanzees that have been found to be naturally infected with *M. leprae* have been shown to develop a lepromatous form of the disease; therefore, green, rhesus and mangabey monkeys have been used in experimental infections. These animals develop disease that resembles the human lepromatous form both clinically and histopathologically (Meyers, W.M. *et al*, .B. *et al*, 1985, 1985; Baskin, G); however, the disease takes about five years to become established. In addition, there are many constraints on using primates for research purposes. These constraints make the use of this model prohibitive for leprosy research.

Although there are no ideal animal models for leprosy research, the mouse and armadillo have been used to study various aspects of leprosy. The mouse granuloma model has been used extensively to study the response of immune cells to infection by *M. leprae* and both animal models have emphasized the bacteria's preference for cooler temperature sites for infection. (Walsh, G. *et al*, 1986).

## **1.4. The Schwann Cell**

*M. leprae* is the only bacterium known to specifically target, infect and replicate within the Schwann cell, the primary support cell of the peripheral nervous system (PNS) (Adams, L.A. and Krahenbuhl, J.L., 1996). The finding of acid-fast bacilli within the Schwann cell is the histopathologic hallmark of leprosy (Clements, B. *et al*, 1996).

### **1.4.1. Schwann Cell Biology**

Schwann cells originate from the neural crest during embryonic development, migrate with extending axons of the nerve into the periphery. The cells then proliferate along the axons, differentiate, ensheath and myelinate axons and then remain quiescent during adult life unless stimulated by nerve damage to aid in the regenerative process (Jessen, K.R. and Mirsky, R., 1998; Li, Rong-hao *et al*, 1996). Schwann cells can first be found in the nerve during development shortly after the outgrowth of axons and before they begin to invade the axon bundle by extending fingerlike processes between individual axons (Gould, R.M. *et al*, 1992).

The initial axonal contact triggers the Schwann cells to proliferate and populate the axons. Larger axons are enveloped first by the Schwann cells which then flatten themselves and exhibit an elongated nucleus orientated longitudinally along the nerve fiber. Eventually, each axon is enveloped by a succession of Schwann cells, each 200 - 2000  $\mu\text{m}$  long, arranged end-to-end to form a continuous sheath until the nerve reaches its target organ (Thomas, P.K. and Ochoa, J., 1984). These axon-Schwann cell units make up individual neurons, which are bundled together to form fascicles, surrounded by a dense fibrous tissue wrapping, the perineurium. Fascicles are further grouped into bundles making up a nerve trunk, the outer covering of which is another layer of fibrous tissue, the epineurium.



After a 1:1 relationship between an individual Schwann cell and axon is achieved, the Schwann cell will begin to elaborate a basement membrane or basal lamina composed of laminin-2, collagen IV, heparin sulfate proteoglycan and entactin/nidogen (Bunge, M.B. *et al*, 1990). The Schwann cell's plasma membrane then begins to wind around the ensheathed axon. After 2-6 turns are completed, compaction begins, the wound plasma membrane begins to differentiate and the myelin sheath begins to take shape and ultimately functions as an insulator around the axon which allows for faster transmission of nerve impulses along the ensheathed axon by preventing the dissipation into the surrounding medium (Garbay, B. *et al*, 2000). The thickness of the myelin sheath is proportional to axon caliber and has been shown to increase as the axon grows.

The myelin sheath is composed of a complex of various proteins (20-30%) and glycolipids (70-80%) that must be produced and then maintained by the Schwann cell within the peripheral nervous system (see **Table 1 for a partial list**). While there are no myelin-specific glycolipids, there are several myelin specific glycoproteins that become detectable with the onset of myelination (Owens, G.C. and Bunge, R.P., 1989; Jessen, K.R. and Mirsky, R., 1991).

Although every Schwann cell has the potential to become a myelinating Schwann cell, myelin will only be produced if the cell comes into contact with certain types of axons, primarily those with a diameter  $> 0.7 \mu\text{m}$  (Webster, H. deF., 1971; Windebank, A.J. *et al*, 1985). The Schwann cell will then be stimulated to produce a basal lamina which is a prerequisite for myelin production (Bunge, M.B. *et al*, 1990).

Non-myelin forming Schwann cells remain associated with multiple smaller axons and extend thin long processes that encircle and separate the smaller axons from one another. The non-myelin forming Schwann cell produces an outer cuff of basal lamina and collagen fibrils

around the ensheathed axon (Ochoa, J. *et al*, 1971; Armati, 1980). Both myelinating and nonmyelinating Schwann cells produce an extracellular matrix which forms a continuous basal lamina and collagen cuff around the axon (Armati, J.P. and Pollard, J.D., 1986).

#### 1.4.2. Schwann Cell Markers

There are several Schwann cell molecules that are useful for identifying Schwann cells, as well as, useful for identifying the Schwann cell phenotype (premyelin, myelinating, or nonmyelin-producing) (**Table 1**). These molecules or Schwann cell markers are often used in immunocytochemical analysis following nerve injury because the relationship between the axon and Schwann cell has been altered and identification of the Schwann cell by morphology alone becomes difficult (Gould, R.M. *et al*, 1992).

S100 immunostaining is routinely used to specifically confirm the identity of the Schwann cells (Brockes, J.P. *et al*, 1979). This calcium binding protein can be found in all Schwann cell phenotypes and may function in stimulating neurite outgrowth and in Schwann cell proliferation (Selinfreund, R.H., Barger, S.W., Welsh, M.J. and Van Eldik, L.J., 1990).

**Table 1. Expression of Schwann cell markers in different Schwann cell phenotypes.\***

Marker	Myelinating Schwann Cell	Nonmyelin-producing Schwann Cell	Short-Term Cultured Cells	Precursors
<b>S100</b>	+	+	+	-
<b>P0</b>	+	-	-	-
<b>MAG</b>	+	-	-	-
<b>GFAP</b>	-	+	+	-
<b>NCAM</b>	+	+	+	+
<b>L1</b>	+/-	+	+	+

\* Gould, R.M. *et al*, 1992

P-zero (P0) is the major myelin protein of the peripheral nervous system, comprising 50-70% of the total protein (Armati, P.J. and Pollard, J.D., 1986). P0 is expressed only by

myelinating Schwann cells and functions as a membrane adhesion molecule to promote and maintain tight compaction of the myelin structure (Lemke, G. and Axel, R., 1985).

Myelin-associated glycoprotein (MAG) accounts for 0.1% of myelin proteins in the peripheral nervous system. MAG on the Schwann cell apparently participates in axonal recognition and adhesion, intermembrane spacing, signal transduction during glial cell differentiation, regulation of neurite outgrowth and the maintenance of axon-myelin integrity (Heape, A. *et al*, 1998). MAG also allows the myelinating glial cell to bind to specific gangliosides on the axonal membrane for adhesion (Collins, B.E. *et al*, 1997; Yang, L.J., 1996).

Glial fibrillary acidic protein (GFAP) is expressed during the precursor or premyelin stage of the Schwann cell but is down-regulated after axonal contact and differentiation (Jessen, K.R. *et al*, 1990; Jessen, K.R. and Mirsky, R., 1991). GFAP is the major protein constituent of glial intermediate filaments (Chen, W.J. *et al*, 1994) and has also been shown to have an important role in Schwann cell/ neuron interactions as well.

Expressed by neurons and Schwann cells, neural cell adhesion molecule (NCAM) and the related neural cell adhesion molecule L1 (L1) both have roles in Schwann cell/axon and Schwann cell/Schwann cell interactions. These surface molecules are selectively expressed by Schwann cells only at sites of contact between their plasma membranes and neurons and contact with other Schwann cells (Martini, R. *et al*, 1994; Armati, P.J. and Pollard, J.D., 1996). Schwann cells that are not in direct contact with another cell, express both molecules on their entire cell surface; however, both are down-regulated upon axonal contact prior to myelination (Garbay, B. *et al*, 2000).

#### **1.4.3. Schwann Cell Demyelination and Nerve Fiber Regeneration**

During nerve injury or degeneration, Schwann cells participate in the disassembling and removal of their own myelin sheath, as well as the degenerating axon by autophagocytosis (Bigbee, J.W., Yoshino, J.E. and DeVries, G.H., 1987; Stoll, G. *et al*, 1989; Reichart, F. *et al*, 1994). Schwann cells dedifferentiate into the premyelin phenotype that allows for proliferation if and when a regenerative response occurs.

Regeneration within the peripheral nervous system following trauma basically recapitulates the developmental process (Fu, S.Y. *et al*, 1997). Schwann cells can both stimulate and mediate nerve tissue regeneration following nerve trauma or injury and are believed to have a critical role in the promotion of axonal process outgrowth (Bixby, J.L. *et al*, 1988). During Wallerian degeneration (a model for nerve degeneration), Schwann cells degrade myelin sheaths, proliferate and re-seed the distal stump (Ide, C., 1996). Trophic factors produced by the Schwann cells stimulate the proximal axons to grow and innervate the distal stump. Schwann cells also serve as scaffolds for the extension of regenerating axons by expressing various adhesion molecules (Bunge, R.P., 1993). Schwann cells then proliferate along the ‘naked’ axons and once again form myelin.

#### **1.4.4. Schwann Cell Marker Expression During Regeneration**

Schwann cell integrin-class extracellular matrix receptors and cell surface adhesion molecules (CAMs), such as, NCAM, L1 and neural-cadherin (N-cadherin) have been shown to have direct involvement in neurite outgrowth promotion, in the Schwann cell /axon relationship, and in myelination (Nieke, J. and Schachner, M. 1985; Martini, R. and Schachner, M., 1988; Cifuentes-Diaz, C. *et al*, 1994). The regulation of these adhesive glycoproteins on the surface of both the neuron and Schwann cell may determine the extent of reinnervation that occurs

following peripheral nerve. It is not known if lymphokines secreted by invading macrophages can stimulate the production or alteration of these molecules (Fu, S.Y. and Goron, T. 1997).

In addition to these specific molecules, transforming growth factor beta-1 (TGF $\beta$ -1) has been shown to be an important mediator of many cellular genes and functions including Schwann cell/neuron interactions and the Schwann cell response to injury or trauma (Garbay, B. *et al*, 2000). Both Schwann cells and neurons have the capacity to secrete TGF $\beta$ -1. Treatment of primary Schwann cells with TGF $\beta$ -1 increases Schwann cell proliferation, increases NCAM expression and promotes the pre- or non-myelin-producing Schwann cell phenotype. When cells are co-cultured with purified neurons, Schwann cells are stimulated to down-regulate TGF $\beta$ -1 expression and in turn express high levels of TGF $\beta$ -1 receptors (Einheber, S., 1995).

When co-cultures are exposed to exogenous TGF $\beta$ -1, many of the effects of axonal contact on the Schwann cell are diminished or altered. There is reduction of axonal-stimulated Schwann cell proliferation, reduced expression of laminin, and defective basal lamina formation resulting in the blockage of myelin formation. Current theory proposes that after nerve trauma or injury TGF $\beta$ -1 promotes the transition of Schwann cells to a proliferating, nonmyelinating phenotype in order to enhance the regenerative response within the nerve. Studies have also shown that TGF $\beta$ -1 added to Schwann cell/neuron co-cultures allows for the Schwann cell to ensheath the axon, but blocks the expression of several key components of the myelin sheath including MAG, P0 and myelin basic protein (MBP) with an accompanied increase in GFAP expression (Mews, M. and Meyer, M., 1993; Guenard, V. *et al*, 1995).

#### **1.4.5. Schwann Cell Culture Techniques**

A variety of *in vitro* cell culture techniques are currently available for the harvest, establishment, maintenance and expansion of Schwann cells from both murine and human

tissues (Casella, Gizelda T.B. *et al* 1996; Kleitman, N. *et al*, 1996). The Brockes' method is a very efficient method for obtaining purified rat Schwann cells from excised, dissociated, sciatic nerves from 1-3 day old rat pups (Brockes, J.P. *et al* 1979). A major benefit of this technique is that the neonatal rat nerve trunk at this stage of development contains relatively few fibroblasts in comparison to Schwann cells. Even though this is the case, after the cells are enzymatically stripped from the sciatic nerve by incubation with collagenase, dispase and trypsin and plated onto plastic, there is still a significant population of fibroblasts within the preparation. However, there are several accepted methods for subsequent fibroblast removal.

Differential adhesion is an efficient method of fibroblast removal that can be employed immediately after harvest and enzymatic digestion of the sciatic nerve (Kreider, B.Q., *et al*, 1981). Briefly, the Schwann cell suspension is allowed to settle on a poly-L-lysine-coated tissue culture surface for 30 minutes with intermittent shaking (gentle rocking of flask) every five minutes. The majority of the fibroblasts quickly bind to the poly-L-lysine-coated surface while most of the Schwann cells remain in suspension. The suspension is then transferred to a new plate leaving the fibroblasts behind.

Another effective means of fibroblast removal from Schwann cell cultures is treatment of cultures with the antimitotic, cytosine arabinose (AraC), shortly after (24 h) the cells are first isolated. A five to seven day treatment of the culture with AraC will remove the majority of fibroblasts. The remainder of the fibroblasts can be removed by complement-mediated lysis using the Thy 1.1 antibody (Lake, P. *et al*, 1979). The Thy 1.1 molecule is expressed on fibroblasts but not Schwann cells.

Once purified of other contaminating cell types (primarily fibroblasts) using a combination of the above techniques, Schwann cells are usually > 90% pure, can be cultured in

the absence of axons and retain full function (Seth Porter, M. *et al*, 1986). In addition, they will dedifferentiate in a similar manner to the way they dedifferentiate during degeneration of the nerve and become similar to the premyelin phenotype. These cells will not assemble a basal lamina nor produce myelin (Bunge, M.B. *et al*, 1990) in the absence of axons. However, when these cells are seeded onto axons of cultured neurons, they will once again associate with axons, ensheath axons and differentiate to form the myelinating and non-myelin-producing phenotypes.

When cultured alone *in vitro*, Schwann cells divide extremely slowly, with a doubling time of roughly 7-10 days in serum containing medium (Kleitman, N. *et al*, 1996; Li, H. *et al*, 1998). Adult Schwann cells associated with an axon *in vivo* do not normally divide, but may be stimulated to proliferate after nerve injury by mitogens derived from ingrowing axons and/or recruited macrophages (Grothe, C. *et al*, 1997).

Mitogens have proven to be an efficient means of expansion for Schwann cell populations *in vitro* (Rahmatullah, M *et al*, 1998; Li, H *et al*, 1998). Expression of many Schwann cell genes, associated with cell replication, is normally regulated by axonal signals. However, Schwann cells can be selectively induced to proliferate *in vitro* in serum containing medium by the addition of forskolin, an activator of adenylyl cyclase, and glial growth factor (GGF, derived from bovine pituitaries) alone or in combination with the recombinant form of GGF, heregulin. Forskolin alone stimulates Schwann cell proliferation; however, the addition of GGF or heregulin more than doubles the proliferative response (Seth Porter, M. *et al*, 1986).

These mitogens grant a competitive edge in proliferation for Schwann cells over the contaminating fibroblasts *in vitro* since fibroblasts-like cells do not proliferate in response to these mitogens. Serum alone allows for some proliferation, but acts synergistically with the

mitogens to produce an increase in Schwann cells numbers greater than that produced by either agent alone (Dong, Ziping *et al*, 1997). Schwann cells produced, sub-cultured, and maintained on mitogens for several months have been shown to be essentially identical to freshly harvested Schwann cells in various secreted proteins and functional capabilities such as association with neurons *in vitro* and the subsequent capacity to myelinate (Seth Porter, M. *et al*, 1986).

Schwann cells treated with mitogens are plated on poly-L-lysine-coated plastic to retain larger numbers of proliferating cells (Seth Porter, M. *et al*, 1986). Mitogen treatment of Schwann cells decreases doubling time to roughly every 2-5 days, depending on whether both GGF and heregulin (2 day doubling) or just GGF (5 day doubling) is used in conjunction with forskolin and serum. However, if the cells are plated on plastic alone, approximately one-half of the cells detach from the substratum during mitosis and are lysed or phagocytized by the remaining cells. When plated on poly-L-lysine, the dividing Schwann cells adhere better to the substrate; and therefore, greater numbers of Schwann cells can be retained and contribute to the forming monolayer population more rapidly than if plated on plastic alone.

Schwann cells initially exposed to forskolin exhibit a slightly altered morphology of a more fibroblast-like broad and extended appearance; but when dissociated with trypsin and replated without forskolin, the cells return to the typical spindle-like morphology for which Schwann cells are known. Schwann cells that have been stimulated by forskolin alone become quiescent within 4 days of forskolin removal, and are capable of being stimulated to proliferate by the reapplication of mitogens. When allowed to proliferate, Schwann cell populations will eventually form whirling confluent monolayers. As a monolayer exposed to mitogens reaches confluent growth, the growth rate of Schwann cells decreases; however, the cells will continue to proliferate and wedge themselves into a more compacted parallel alignment.



Transformed glial cell lines such as 33B (Band, A.H. *et al*, 1986) and S16 (Sasagasaki, N. *et al*, 1999) are also commonly used in Schwann cell research. These transformed cell lines are easily maintained on tissue culture medium. However, the cells have an extremely rapid growth rate (24 h) as well as a loss of typical Schwann cell functional characteristics, such as the ability to associate and interact with neurons. These properties render these cells unsuitable for studies to define the effects of long-term infection on Schwann cells or on Schwann cell/neuron interactions.

#### **1.4.6. Purification of Neurons**

Within the day 15 developing rat embryo (E15), many dorsal root ganglia are positioned on both sides of the spinal chord (Kleitman, N., 1996). Neurons purified from rat embryonic dorsal root ganglia (DRG) have the potential to produce large axons that normally course through both the central and peripheral nervous systems. These neurons are capable of inducing myelination either by Schwann cells or oligodendrocytes, the myelinating support cell of the central nervous system. DRG neurons or their axons are also capable of stimulating upon contact Schwann cell proliferation, extracellular matrix or basal lamina deposition, the expression of myelin components and eventually the production and maintenance of the myelin sheath (Obremski, V.J. and Bunge, M.B., 1995).

An efficient method to purify neurons is described by Wood (1976). Briefly, the DRG are harvested from the spinal column of E15 rats, dissociated, and plated onto a rat-tail collagen substrate. These cells are then subjected to two to three rounds of fluorodeoxyuridine (FdU), an antimitotic that essentially removes all non-neuronal cell types from the culture (Wood, P.M., 1976). If non-neuronal cells (fibroblasts), are not effectively removed from the culture at this stage of isolation, an ensheathing cellular mantle or *in vitro* version of the perineurium will form

over the culture preventing direct view and access to the Schwann cell-axon unit (Mithen, F.A. *et al*, 1982). Fibroblasts are never entirely eliminated from the culture, but should be diminished in population as much as possible prior to the addition of Schwann cells. After approximately three weeks, the neuronal cells should be relatively free of other contaminating cell types and can then be “seeded” with purified Schwann cells.

#### **1.4.7. Schwann Cell/Neuron Co-cultures**

In order to develop an *in vitro* model that is representative of the peripheral nervous system (PNS) and therefore contains the myelinated Schwann cell phenotype, Schwann cells must be co-cultured with purified neurons (Bunge, M.B. *et al*, 1990). DRG neurons or their axons are capable of stimulating upon contact Schwann cell proliferation, extracellular matrix or basal lamina deposition, the expression of myelin components and eventually the production and maintenance of the myelin sheath (Obrebski, V.J. and Bunge, M.B., 1995). In order to produce the basal lamina required for myelination, the Schwann cell must have direct contact with an axon and a collagen substrate along with ascorbate and serum in the medium. Serum free or defined co-culturing conditions can be utilized if laminin is added to the medium.

To produce myelinating Schwann cell/neuron co-cultures, purified primary Schwann cells are seeded directly onto purified neurons cultured on rat tail collagen. Within 24 h the Schwann cells will adhere and align with the axons. These cells will then be stimulated by axonal contact to proliferate and populate the neurons. After the co-cultures are sufficiently populated with Schwann cells and the cells are “poised” for myelination, ascorbic acid can be added to the culture medium to stimulate the process of myelination. Ascorbate permits the Schwann cells to produce the triple helical collagens necessary for the proper formation of the basal lamina that is a necessary prerequisite for the production of myelin by the Schwann cell.

The establishment and development of these co-cultures generally requires that they be maintained for a period of weeks to months to allow for the full development of the cellular interactions resulting in myelination (Kleitman, N., 1996). In addition, once established, the myelinating co-cultures can be maintained on myelinating feed for weeks to months (personal communication with Dr. Pat Wood, Miami Project to Cure Paralysis, University of Miami Medical School, Miami, FL).

## **1.5. Schwann Cell and *M. leprae* Interactions**

### **1.5.1. Neuropathology**

The neuropathology of leprosy is poorly understood (Shetty, V.P. *et al*, 1994; Spierings, E. *et al*, 2001; Job, C.K., 1971). Based on histopathological observations of patient nerve biopsies, *M. leprae* appear to be relatively non-toxic. However, its presence within the nerve or Schwann cell *in vivo* may alter function and stimulate an immune or inflammatory bystander response to infection within the highly sensitive nerve compartment resulting in demyelination and Schwann cell and axonal loss.

Peripheral nerve damage occurs throughout the host immunologic spectrum of response. This damage has been attributed to overwhelming bacterial proliferation as seen in the lepromatous form of the disease as well as immunologic response to few bacteria or bacterial antigens as seen in the tuberculoid form of the disease (Adams, L.B. and Krahenbuhl, J.L., 1996). There appears to be a common mechanism of nerve damage in the early stages of both tuberculoid and lepromatous leprosy (Shetty, V.P. *et al*, 1988). Demyelination and the destruction of Schwann cells result in nerve damage potentially leading to sensory loss and disfiguration of afflicted areas.

### **1.5.2. *In vitro* Models to Study *M. leprae*/Schwann Cell Interactions**

The direct effects of *M. leprae* infection on Schwann cells have been investigated *in vitro* using various models (Steinhoff, U. *et al*, 1991; Mistry, Y. *et al*, 1992; Mukherjee, R. and Antia, N.H., 1985; Singh, N. *et al*, 1998). Many of these models have used *M. leprae* preparations that had been collected from a variety of sources including: patient biopsies, armadillo tissues and mouse foot pad tissue. Viability of the bacteria in these preparations was not verified prior to or during the infection of the Schwann cells. In some studies, frozen and thawed and heat-killed bacteria were used (Steinhoff, U. *et al*, 1989; Singh, N. *et al*, 1996; Maeda, M. and Narita, M., 1987; Singh, N. *et al*, 1997). In addition to different *M. leprae* preparations used for these investigations, different Schwann cell populations were used. Schwann cells harvested from mice, rats, and human tissues as well as transformed, glial cell lines such as 33B (Band, A.H. *et al*, 1986) and Schwannoma cell lines such as S16 (Sasagasaki, N. *et al*, 1999) have been commonly used to study Schwann cell/*M. leprae* interactions. This has resulted in varied results and conflicting conclusions as to the response of Schwann cells to infection *in vitro*.

### **1.5.3. Molecular Targeting and Phagocytosis**

Recent research has shown that the trisaccharide unit of the phenolic glycolipid-1 (PGL1) and *M. leprae* laminin-binding protein 21 (ML-LBP21) on the surface of irradiated *M. leprae* specifically binds the G domain of the native laminin  $\alpha$ 2 chain on the basal lamina of rat Schwann cell axon units (Ng, V. *et al*, 2000). A correlating study using human Schwann cells also found that *M. leprae* specifically binds a major phosphorylated glycoprotein of the peripheral nerve with biochemical characteristics similar to those reported for P0 of peripheral nerve myelin (Suneetha, L.M. *et al*, 1997). By targeting and facilitating the crossing of the basal

lamina, these *M. leprae* cell wall-associated proteins and glycolipids allow for bacterial invasion of the Schwann cell within the peripheral nerve.

Schwann cells cultured in the absence of neurons, fail to assemble an organized basal lamina, but will nonspecifically phagocytize bacteria or beads placed in culture with them (Band, A.H. *et al*, 1986a, 1986b). Phagocytosis of *M. leprae* by Schwann cells is significantly reduced by heat killing or formalin-saline treatment of the bacteria (Mukherjee, R., 1986). Steinhoff *et al* (1989) have demonstrated using frozen-thawed *M. leprae* that Schwann cells are capable of phagosome-lysosome fusion after phagocytosis which may indicate the ability of Schwann cells to degrade phagocytized *M. leprae*.

#### **1.5.4. Collagen and Basal Lamina**

Histopathological observations of nerve biopsies taken from patients indicate that during the early stages of infection, there is increased collagen deposition. Experiments using Swiss Webster mice inoculated with *M. leprae* (viability of inoculum was not reported) have shown an increase in collagen deposition (Singh, N. *et al*, 1998). This correlates with *in vitro* studies of infected Schwann cells that showed increased synthesis of collagen types I, III and IV as a result of infection (Singh, N. *et al*, 1997). Since Schwann cells are responsible for the majority of collagen production within the peripheral nerve, these data suggested that alterations of collagen deposition during *M. leprae* infection occurred in the absence of inflammatory cells and are, therefore, similar to the early stages of nerve involvement.

#### **1.5.5. Adhesion Molecules**

*In vitro* infection of Schwann cells has been shown to affect the expression of adhesion molecules such as NCAM (Singh, N. *et al*, 1996). Sullivan *et al* (1991) demonstrated that the expression of adhesion molecules within leprosy lesions was related to the level of cell-mediated

immunity against *M. leprae*. Intracellular adhesion molecule (ICAM), which promotes adhesion and signaling with lymphocytes, was found to have higher expression within tuberculoid lesions paralleled with higher lymphokine levels while lepromatous lesions were typically characterized by the absence of ICAM expression (Wong, L. *et al*, 1989).

#### **1.5.6. Electron Microscopy Studies**

Several electron microscopic studies performed on human leprosy patient biopsies have reported alterations in myelin morphology. Although leprosy may result in progressive neuropathy involving both motor and sensory nerves (Job, C.K., 1971), studies did not reveal a consistent pathology. However, different stages and/or degrees of pathology were observed, which may vary according to duration and level of infection, spectrum nature of the disease manifestation as well as duration and types of treatment received by the patient.

Job (1971) reported that the earliest identifiable leprosy lesions showed slight proliferation of Schwann cells within the nerve bundle as well as obvious edema of the tissue without alteration of myelin or axons. Schwann cells with larger numbers of *M. leprae* exhibited vacuolation without alteration of the nerve itself, though later stages exhibited foamy Schwann cells along with ballooning and fragmentation of axons and extensive demyelination. Sometimes the entire nerve bundle was not involved, but rather a portion seemed to be preserved from damage. Later stages of infection demonstrated entire nerve bundles were replaced by fibrous tissue with a gradual reduction in bacillary load. Therefore, it was concluded that the destruction of axons in lepromatous leprosy is due primarily to the irreversible damage to Schwann cells infected with *M. leprae* which is then further aggravated by cellular infiltration and increased collagen and proliferation of perineurial cells.

While some atrophic fibres with small axons from lepromatous and tuberculoid lesions appeared to have normal myelin, some nerve fibres exhibited “loose” myelin, abnormal periodicity and little or no evidence of compression as judged by the density of axonal organelles (Jacobs, J.M. *et al*, 1987a). Another related study reported leprosy nerves with focal regions of total demyelination often occurring in relation to cellular infiltration but without evidence of macrophage removal of myelin (Jacobs, J.M. *et al*, 1987b).

Though edema was observed in nerves within lesions in this study, few pathological features were common. Some of the demyelination could have been secondary to axonal changes, though no direct evidence of this was obtained in this study (Jacobs, J.M. *et al*, 1987a). Some nerve fibers in lepromatous nerves seemed of small size relative to myelin sheath thickness, yet there were no obvious correlations of myelin structure abnormality associated with relative changes in axon size. Degenerating axons were rarely observed in this study, although one example of a degenerating axon ensheathed by a myelinated Schwann cell of normal appearance was found.

#### **1.5.7. Schwann Cell/Neuron Co-cultures**

Experiments using Schwann cell/neuron co-cultures have shown that Schwann cells associated with axons prior to myelin formation were capable of phagocytizing freshly harvested and heat killed *M. leprae*. However, once infected, the Schwann cells were unable to associate with or produce myelin around axons (Mukherjee, R. *et al*, 1980a & 1980b). In these studies bacilli were observed in Schwann cells and fibroblasts, but not within neuronal cell bodies or axonal processes. It was also reported that Schwann cell phagocytosis was influenced by the Schwann cell's level of axonal association. Schwann cells that were not associated with or partially associated with an axon were observed to have more bacilli than Schwann cells in

intimate association with the axon (Mukherjee, R. *et al*, 1980a & 1980b). These partially or non-axonally associated infected Schwann cells failed to associate with axons even after 3-4 weeks in culture under favorable conditions. These results suggested that infected Schwann cells may not be able to participate in regeneration but may continue to survive for a prolonged period of time. It was therefore concluded that Schwann cells merged with axons are less phagocytic or gradually lose their phagocytic activity after myelin secretory function has been initiated. Other reports have suggested that the *in vitro* myelinated Schwann cell is a quiescent cell that becomes phagocytic only after injury and degenerative changes within the nerve (Palmer, E., Rees, R.J.W. and Weddell, G., 1961; Singer, M. and Steinberg, M.C., 1972).

Schwann cell/neuron cultures labeled with  $^3\text{H}$ -thymidine at the time of infection have shown a marked reduction in DNA synthesis suggesting that Schwann cells infected with *M. leprae* have reduced proliferative capabilities (Mukherjee, R. *et al*, 1980b). This could in turn severely limit their ability to proliferate along axons and subsequently produce myelin. Observations revealed that most of the  $^3\text{H}$ -thymidine was taken up by cells that had not phagocytized *M. leprae*, suggesting that intracellular bacilli inhibit DNA synthesis. Heat inactivated *M. leprae*-infected cells acquired  $^3\text{H}$ -thymidine comparable to that of uninfected cells which suggested that dead *M. leprae* could not produce the same effect. These results suggested that infection of Schwann cells with viable *M. leprae* results in decreased proliferative capacity of these cells and, therefore, decreases the Schwann cell's capacity to participate in the nerve regenerative processes.

Protein production of Schwann cell/neuron co-cultures infected with freshly harvested *M. leprae* was also evaluated in this study (Mukherjee, R. *et al*, 1980b). Results of this study



suggested that *M. leprae* infection did not result in detectable alteration of protein synthesis during infection up to 12 days post infection.

The bacterial preparations for the above experiments were obtained from nodules from untreated leprosy patients (Mukherjee, R. *et al*, 1980b). The viability of the *M. leprae* preparations used in these experiments was not analyzed and is, therefore, in question. This is even obvious to the authors who state that: “the viability within the Schwann cells can only be assumed since it was not directly proved” (Mukherjee, R. *et al*, 1980b). Mukherjee, R. and Antia, N.H. (1985) reported that *M. leprae* multiply within Schwann cell/neuron co-cultures. This was measured by analyzing the incorporation of radiolabeled thymidine within Schwann cell preparations and by the use of acid fast-stained bacterial counts for up to 28 d post infection at 37<sup>0</sup> C. In this system, bacilli were reported to double every seven days; however, no direct viability analysis such as mouse footpad inoculation was used.

Therefore, even though there appears to be a wealth of information concerning the effects of *M. leprae* on Schwann cells using various *in vitro* models, the majority of this research is based on infection of these cells with dead *M. leprae*. Since the viability of a pathogen can have a major impact on the outcome of infection, it is essential that a new model be developed that maintains the viability of *M. leprae* for experimental studies.

## **1.6. Current Research Objectives**

Both of the WHO and NIH research priorities for leprosy projects demonstrate the need for heightened work in the area of understanding the neuropathology of leprosy (Fox, J.L., 2001). The goal of this research effort is to gain information concerning the process of progressive nerve damage that will ultimately lead to the development of new tools for early diagnosis of progressive nerve damage and improved therapies for treatment early in the course

of disease. The potential benefit of this is the global reduction of neuropathological morbidity associated with leprosy, thereby, reducing the prevalence of disabilities and providing a better quality of life for leprosy patients.

However, the use of various models to study *M. leprae*/Schwann cell interactions has lead to conflicting reports in the literature. These interactions will not be understood until an appropriate and consistent model of infection is identified and employed. Therefore, a major focus of research should be to develop a representative model to define the direct effects of *M. leprae* infection on Schwann cells early in the course of disease development. In particular, a model is needed to define the effects of viable *M. leprae* on the Schwann cell's ability to interact appropriately with axons and produce and maintain myelin sheathe architecture.

## **CHAPTER 2.**

### **DEVELOPMENT AND CHARACTERIZATION OF AN IMPROVED MODEL FOR STUDYING *MYCOBACTERIUM LEPRAE*/ SCHWANN CELL INTERACTIONS**

#### **2.1. Introduction**

Presently there are between 1-3 million leprosy patients in the world that are physically disabled as a result of damage to peripheral nerves and the attendant sensorimotor loss (World Health Organization, 1998). It is estimated that one fourth of the 690,000 newly diagnosed patients each year will suffer from irreversible nerve damage, possibly resulting in the classic hand and foot deformities of leprosy and concomitant disabilities as a long-term consequence of infection with *Mycobacterium leprae* (World Health Organization, 1995 and 2000).

*M. leprae* is an obligate intracellular parasite which is unique among bacterial pathogens in its ability to invade the peripheral nervous system and is the leading cause of nontraumatic neuropathies worldwide. Despite this, the specific effects of *M. leprae* on nerves and the events which result in nerve destruction are not well understood.

The Schwann cell, the principle support cell in the peripheral nervous system, appears to be the major target of *M. leprae* within the peripheral nervous system (Lumsden, C.E., 1959; Job, C.K., 1989; Rambukkana, A., 1997). As a long term consequence of infection and immunologic response, Schwann cells are ultimately destroyed or functionally impaired in damaged nerves (Job, C.K., 1971). Atrophy of myelinated fibers and secondary demyelination has been shown to occur as result of infection, sometimes even in the absence of evidence of inflammation or acid-fast bacilli (Shetty, V.P. *et al*, 1994; Jacobs, J.M. *et al*, 1987).

The mechanism by which *M. leprae* enter the nerve to reach the Schwann cells is under investigation (Scollard, D.M., 2000); however, the mechanism of binding of *M. leprae* to the Schwann cell has been recently elucidated (Rambukkana, A., 2000). Once *M. leprae* are

adherent to the Schwann cell surface, they are ingested (Ng, V. *et al*, 2000). However, the Schwann cell appears incapable of destroying this intracellular parasite (Kaplan, G. and Cohn, Z.A, 1986). There are conflicting reports concerning the effects of *M. leprae* infection on Schwann survival and function and no comprehensive study has been performed to determine the fate of *M. leprae* within Schwann cells. It is, therefore, conceivable that long-term infection of Schwann cells and multiplication within these cells may lead to damaging effects on these cells, resulting in loss of nerve function prior to stimulation of the immunologic response (Job, C.K., 1971).

One potential reason for these conflicting reports is that no standardized model has been used for these studies. For example, *M. leprae* have been harvested from various sources and irradiated, frozen and thawed or held at various temperatures prior to infection of Schwann cells (Steinhoff, U. *et al*, 1991; Mistry, Y. *et al*, 1992; Mukherjee, R. and Antia, N.H., 1985; Singh, N. *et al*, 1998). Truman and Krahenbuhl (2001) have recently documented that the viability of *M. leprae*, harvested from a variety of sources, varies greatly. In addition, they demonstrated that many routine laboratory practices, such as storage in 7H12 medium at 37<sup>0</sup> C, rapidly reduce the viability of *M. leprae* in these preparations (Truman, R.W. and Krahenbuhl, J.L., 2001). Therefore, it does not appear that viable *M. leprae* were used in the majority of previous experiments to study Schwann cell/*M. leprae* interactions.

Since the viability of a bacterium can greatly influence the experimental outcome, it is imperative that an improved *in vitro* model be developed which maintains the viability of *M. leprae* without affecting Schwann cell and neuron survival or function. Storage of *M. leprae* at 33<sup>0</sup> C in the 7H12 medium has been shown to maintain the viability of *M. leprae* for weeks (Truman, R.W. and Krahenbuhl, J.L., 2001). These data suggest that an improved model should

include infection of Schwann cell cultures or Schwann cell/neuron co-cultures with freshly harvested *M. leprae* and maintenance of these cultures at 33<sup>0</sup> C. However, to date, no comprehensive studies have been conducted to determine the effects of this temperature on *M. leprae* viability within Schwann cells or on Schwann cell survival and function. Therefore, the effects of 33<sup>0</sup> C on *M. leprae* viability within Schwann cells and on Schwann cell survival, Schwann cell gene expression, and the ability of these cells to associate and interact to form myelinated co-cultures were determined.

Results of the present study demonstrated that culturing Schwann cell at 33<sup>0</sup> C did not appear to affect their survival rates or functional capabilities. In addition, the metabolic activity of *M. leprae* was greater in these cells than those held at 37<sup>0</sup> C and infection had greater effects on Schwann cells cultured at 33<sup>0</sup> C than at 37<sup>0</sup> C. Therefore, an improved model for studying the effects *M. leprae* infection on Schwann cells and Schwann cell/neuron interactions that retains *M. leprae* metabolic activity without the apparent loss of Schwann cell and neuron function and survival has been developed.

## **2.2. Materials and Methods**

### **2.2.1. Bacteria**

*M. leprae* Thai-53 were passaged and purified from the hind foot pads of nude BALBc (*nu/nu*) mice, using a previously described method (Adams, L.B. *et al*, 1997). These bacteria were suspended in 7H11 medium containing albumin and 50 µg/ml ampicillin and incubated for 3 h at 37<sup>0</sup> C. The bacterial pellet was obtained by centrifugation (10,000 x g for 5 min) and resuspended in D-10 medium consisting of Dulbecco's Minimal Essential Medium (Life Technologies Inc., Rockville, MD) with 10 % heat-inactivated fetal calf serum (Hyclone Laboratories, Inc., Logan, UT) and 50 µg/ml gentamycin (Sigma Chemical Company, St. Louis,

MO). Bacterial counts were obtained using acid fast staining (BBL<sup>7</sup> TB Ziehl-Neelsen Kit, Becton Dickinson Microbiology Systems, Cockeysville, MD).

The viability of the bacterial preparations was determined by radiorespirometry using the BACTEC 460 system, (Becton Dickenson) using a previously described protocol (Franzblau, S.G., 1992). All bacterial preparations were analyzed for the presence of contaminants by culture on blood agar, thioglycolate broth, trypticase soy broth, Lowenstein Jensen and 7H11 slants (Life Technologies). Bacteria exposed to  $1 \times 10^6$  rad gamma irradiation in a Shepherd Model 484 irradiator (J. L. Shepherd and Associates, Fernando, CA) using a previously described protocol (Adams, L.B. *et al*, 2000) were used as a >dead= control for all experiments.

### **2.2.2. Schwann Cell Cultures**

Schwann cells were purified from the sciatic nerves of neonatal Holtzman outbred rats (HsdHot:Holtzman<sup>J</sup> SD, Harlan, Indianapolis, IN) using a modified Brookes' method (Brookes, J.P. *et al*, 1979; Kleitman, N. *et al*, 1996). Briefly, sciatic nerves were dissected from 1-3 day old rats and dissociated with 0.1% collagenase (Life Technologies) and 0.5% protease (dispase) (Sigma) final concentration in L-15 medium (Life Technologies) and incubated for 30 min at 37<sup>0</sup> C. This preparation was then treated with a final concentration of 0.05% trypsin (Sigma) and 0.2% EDTA (Sigma) (trypsin-EDTA), incubated at 37<sup>0</sup> C for 30 min with agitation and resuspended in D-10. The cell suspension was briefly triturated and then passed through a 70 µm nylon filter (Becton Dickinson Labware, Franklin Lakes, NJ) in order to remove remaining large tissue fragments and obtain a single cell preparation. Schwann cells were enriched by differential adhesion using a previously described method (Kreider, B.Q. *et al*, 1981) and further purified by incubation for 5-7 d in D-10 medium containing  $10^{-5}$  M cytosine  $\beta$ -D-arabinofuranoside (AraC)(Sigma).

Schwann cells were then dissociated from the tissue culture flask using trypsin-EDTA in Hanks balanced salts solution, and further enriched prior to replating by complement-mediated lysis by incubation for 30 min each with anti-Thy-1.1 and sterile filtered rabbit complement (Accurate Chemical and Scientific Corporation, Westbury, NY) at 37<sup>0</sup> C with agitation (Brookes, J.P. *et al*, 1977). Anti-Thy1.1 antibodies were previously purified in our laboratory from T11D7e2 mouse-mouse hybridoma cell line (ATCC# T1B103) (American Type Culture Collection, Rockville, MD) as specified by the ATCC and stored at -70<sup>0</sup> C until use.

Schwann cells were sub-cultured for no more than five passages by plating in poly-L-lysine (PLL, Sigma)-coated tissue culture flasks (Corning, Corning, NY) in D-10 at 37<sup>0</sup> C in 5% CO<sub>2</sub> until monolayers were confluent. In order to pre-coat tissue culture surfaces, poly-L-lysine hydrobromide (Sigma) was diluted to 2 mM in sterile H<sub>2</sub>O and incubated at room temperature for 30 min on the culturing surface. The culturing surface (flask or well plate) was rinsed 2-3 times with sterile H<sub>2</sub>O. Schwann cells were dissociated with trypsin-EDTA and replated onto PLL-coated 13 mm plastic Thermanox cover slips (Nunc, Rochester, NY) or in 24-well tissue culture dishes (Corning) at a density of 1 x 10<sup>5</sup>/well in D-10, and incubated at 37<sup>0</sup> C in 5% CO<sub>2</sub> until they reached a confluent monolayer. Cultures were incubated for 24 h at either 33<sup>0</sup> C or 37<sup>0</sup> C in 5% CO<sub>2</sub> prior to infection with *M. leprae*.

To produce large numbers of Schwann cells for use in established co-culture experiments, Schwann cells were mitotically-expanded using characterized Schwann cell mitogens. To accomplish this, Schwann cells were purified as described above then plated into 25 cm<sup>2</sup> flasks with D-10 medium containing the Schwann cell mitogens;  $\beta$ -heregulin (2.5 nM), (a kind gift of Genetech, San Francisco, CA), forskolin (2  $\mu$ M)(Sigma), and bovine pituitary extract

(20 µg/ml)(Biomedical Technologies, Stoughton, MA). Cells were maintained until monolayers produced the typical 'swirled' morphology.

### **2.2.3. Purity and Viability of Schwann Cell Cultures**

The purity of Schwann cell cultures was determined by immunostaining analysis for the presence of the Schwann cell marker, S100 cell surface protein (Pelc, S. *et al*, 1986). This was accomplished using anti-S100 antibody (Sigma, IgG fraction of antiserum developed in rabbit), the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA), the AEC Chromagen Kit (Biogenex, San Ramon, CA) and a previously described protocol (Job, C.K. *et al*, 1990). Cultures were considered pure when fibroblast contamination was  $\leq 5\%$  the total cell population. Trypan blue dye exclusion analysis (Life Technologies) was used to determine the viability of these cell preparations.

### **2.2.4. Neuron Cultures**

Neurons were dissociated and purified from dorsal root ganglia (DRG) harvested from day 15 embryonic rats using a previously described protocol (Kleitman, N., 1996). Briefly, 1.5 DRG were suspended in 100 µl Neurobasal media (NB1)(Life Technologies) containing 1% heat inactivated fetal calf serum, crude nerve growth factor (10 µl/ml in NB1) (prepared in our lab via a personal communication A. Gomez, Miami Project to Cure Paralysis, University of Miami School of Medicine, Miami, Fl) and B27 supplement (1 ml/ 50 ml NB1) (Life Technologies). This suspension was plated into 6-well tissue culture plates, pre-coated with rat tail collagen produced in our laboratory according to a previously described protocol (Kleitman, N. *et al*, 1996). For some experiments, 0.5 DRG suspended in 25 µl NB1 were plated onto 13 mm Thermanox coverslips (Nalge Nunc International, Naperville, IL) pre-coated with rat tail collagen in 24-well plates. Cultures were subjected to three weekly rounds of antimitotic



treatment using NB1 with 4  $\mu$ M fluorodeoxyuridine (Sigma) after which the cultures were visually analyzed for purity using phase contrast inverted microscopy and were considered pure if there were < 5% fibroblast contamination. All cultures were incubated at 37<sup>0</sup> C until axonal spouts extended from neurons and thickened (3-4 weeks).

### **2.2.5. Schwann Cell/Neuron Co-cultures**

When the Schwann cell monolayers had a ‘swirled’ morphology they were considered ready for seeding onto purified neurons. The monolayers were dissociated using 0.05% trypsin-EDTA, resuspended in D-10/NB1 (1:1) and approximately 1 ml containing  $5 \times 10^4$  Schwann cells was seeded onto purified neurons in each well of a 24- well plate. For some experiments  $3-5 \times 10^5$  cells per 3 ml were seeded on neurons in each well in a 6-well plate.

Co-cultures were then incubated at 33<sup>0</sup> C or 37<sup>0</sup> C in 5% CO<sub>2</sub>. After Schwann cells had attached to and proliferated along axons for approximately 12 days, myelination was induced in these cultures by addition of freshly prepared, filter sterilized, aqueous ascorbic acid (1% final concentration)(Life Technologies) as previously described (Kleitman, N. *et al*, 1996).

### **2.2.6. Infection of Schwann Cells and Co-cultures**

To determine the effects of *M. leprae* infection on cultured Schwann cells and the effects of Schwann cells on *M. leprae* viability, Schwann cells were infected at a multiplicity of infection of 100 *M. leprae* per Schwann cell (MOI = 100) and exposed to these bacteria for 48 h at either 33<sup>0</sup> C or 37<sup>0</sup> C in 5% CO<sub>2</sub>. At this time, the cells were considered infected (0 d post-infection). Medium was removed, monolayers were washed with D-10, and the cells were incubated up to 21 days post infection. Media was replenished twice weekly.

Schwann cells were dissociated with trypsin-EDTA at 4 and 12 d post infection with *M. leprae*. Approximately  $5 \times 10^4$  Schwann cells in 1 ml of D10/NB1 (1:1) were seeded onto

purified neurons in 24-well plates and cells were allowed to attach, align and proliferate along axons. Co-cultures were induced to myelinate as described above (**Section 2.2.5.**).

To determine the effects of *M. leprae* on intact, myelinated Schwann cell/axon units, **2** of myelinated co-cultures (**Section 2.2.5.**) were shifted to 33<sup>0</sup> C for 24 h prior to infection. Co-cultures were then infected with *M. leprae* at an MOI = 100. Cultures were incubated for 48 h, washed with D10 and maintained at either temperature up to 30 d post infection. Media was changed three times per week. Co-cultures were checked at various time points post-infection for bacterial contamination by streaking culture supernatants onto blood agar plates and incubating at 37<sup>0</sup> C.

#### **2.2.7. *M. leprae* Viability in Schwann Cells**

The viability of *M. leprae* in Schwann cells was determined indirectly using a modification of the Buddemeyer radiorespirometry method (Franzblau, S.G. *et al*, 1992). Briefly, media was removed from wells containing infected Schwann cells and cells were lysed by the addition of 400 µl of 0.1 N NaOH (Sigma) in sterile H<sub>2</sub>O. Next, 300 µl of the cell lysate was transferred to a Ashorty® vial (Wheaton, Millville, NJ) containing 4 ml BACTEC 12B Middlebrook 7H12 medium containing C<sup>14</sup> palmitate (Becton Dickinson), 50 µg/ml ampicillin (Sigma), 0.04% citric acid (Sigma) and 2-5mg/ml amphotericin B (Sigma). ‘Shorty’ vials were then placed in Poly-Q-scintillation vials (Beckman Instruments, Brea, CA) containing a 2 cm x 4 cm strip of Whatman #42 filter paper (Whatman International Ltd., Maidstone, England) which had been dipped into scintillation fluid mixture as previously described by Franzblau, S.G. (1989). The vials were incubated at 33<sup>0</sup> C for 7 d, and then analyzed for oxidation of palmitate by measuring the release of radiolabelled CO<sub>2</sub> using a Liquid Scintillation Counter (Beckman Scientific Instruments, Inc., Fullerton, CA). Viability was reported as counts per minute (CPM).

### **2.2.8. Acid Fast Thick Sections and Electron Microscopy**

The phagocytic index and percent of cells infected with *M. leprae* in Schwann cell cultures were determined using acid fast thick section methodology as previously described (Luderschmidt, C., 1987). Thin sections were obtained from the same blocks, processed for transmission electron microscopy using previously described protocols (Bozzola, J.J. and Russell, L.D., 1992) and images were obtained using a Phillips 410 transmission electron microscope (Phillips Manufacturing). Schwann cell monolayers and co-cultures were prepared for scanning electron microscopy using standard procedures (Bozzola, J.J. and Russell, L.D., 1992; Hayat, M.A., 1981) and viewed on a Cambridge Stereoscan 150 scanning electron microscope (Cambridge).

### **2.2.9. Purification of RNA and Reverse transcriptase-PCR**

To determine the effect of temperature on Schwann cell gene expression, the level of expression of several genes encoding markers typically expressed in short-term cultured Schwann cells were analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR). To accomplish this, total RNA was purified from individual wells containing Schwann cell monolayers. The medium was removed and cells were lysed by the addition of 300  $\mu$ l guanidinium HCl (RNAgents Total RNA Isolation System, Promega, Piscataway, NJ). RNA was extracted from these cell lysates using the manufacturer's recommendations and stored at -70<sup>0</sup> C until used.

Total RNA was converted to cDNA using the Advantage J cDNA Polymerase Mix and Advantage J RT for PCR Kit (Clontech, Palo Alto, CA), as recommended by the manufacturer. Primers sequences and optimal PCR conditions for amplification of Schwann cell genes encoding glial fibrillary acidic protein (GFAP) and neural cell adhesion molecule (NCAM) were

obtained from Knittel *et al* (1996). Primers and PCR parameters for transforming growth factor-beta 1 (TGF- $\beta$ 1) and the house keeping gene, glyceraldehyde 3-phosphate dehydrogenase (G<sub>3</sub>PDH) were obtained from Clontech (Palo Alto, CA). Primers and PCR parameters for N-cadherin, Intercellular adhesion molecule (ICAM) and L1 were initially designed from cDNA sequences obtained from the NCBI information resource database using Omiga<sup>TM</sup> 2.0 DNA and Protein Sequence Analysis software (Oxford Molecular LTD, Oxford, UK). All primers concentrations and PCR parameters were optimized in our laboratory to verify sensitivity to template concentration and prevent product saturation (**Tables 2.1 and 2.2**). In order to provide a positive control for Schwann cell gene transcripts analyses and primer optimization, cDNA was prepared from the sciatic nerve of 17 d old rat pups and used in parallel reactions with experimental cDNA.

Sample cDNA (5 $\mu$ l) for each sample were added to reactions, amplified by PCR and 15 $\mu$ l of the resultant amplicons were separated by gel electrophoresis on 2 % NuSieve/SeaKem agarose gels (FMC BioProducts, Rockland, ME) and visualized by UV transillumination of ethidium bromide-stained gels using a Gel-Doc 1000 (BioRad Systems, Hercules, CA). Semi-quantitative analysis of the gene expression of various Schwann cell markers was performed by scanning photographic images of gels into a Bio Image7 System (Bio Image7 Products/Millipore, Ann Arbor, MI ) using a 3CX scanner (XRS X-ray Scanner Corporation, Torrence, CA). DNA band intensity was converted into integrated optical density units (IOD) using Visage Electrophoresis Gel Analysis Whole Band Analysis software (Bio Image7 Products. Millipore). The data was normalized by dividing the IOD template for each sample by the IOD of G<sub>3</sub>PDH, the housekeeping gene transcript and reported as normalized integrated optical density (nIOD) values.

**Table 2.1.** Primer sequences for use in polymerase chain reaction (PCR) semiquantitative analysis of cDNA collected from samples.

Schwann Cell Marker	Primer Sequence
G3PDH	Clontech Catalog # 5405-1
GFAP	5'ACTGCAGGCCTTGACCTG3' 5'GATCACCTGCCCATCAGG3'
NCAM	5'AGAACATCCCTCCCAGCC3' 3'GCGTTGTAGATGGTCAGGGT3'
TGFβ1	Clontech Catalog # 5501-1

**Table 2.2.** Polymerase Chain Reaction (PCR) parameters for primer sets (Table 2.1) used for semiquantitative reverse transcription-PCR analysis. All sets were denatured at 94<sup>0</sup> C for 7 minutes; then cycled at 94<sup>0</sup> C, annealing temperature and 72<sup>0</sup> C for the time parameters specified (respectively); and finally placed at 72<sup>0</sup> C for 10 minutes for extension.

Primer	Primer Concentration	Annealing Temperature	Number of Cycles	Cycling Parameters (minutes)	Anticipated Fragment Size
G3PDH	20μM	64 <sup>0</sup> C	25	1, 1, 1	452bp
GFAP	10μM	62 <sup>0</sup> C	35	1, 1, 1	340bp
NCAM	10μM	60 <sup>0</sup> C	35	1, 1, 1	280bp
TGFβ1	20μM	60 <sup>0</sup> C	28	0.5, 0.5, 0.5	525bp

## 2.2.10. Statistical Analysis of Data

Most experiments were performed in triplicate and data were expressed as the mean and standard deviation of at least three replicates per experiments. Data were either analyzed by student t-test or by SAS (Statistical Analysis Systems, Cary, NC) analysis of variance in a factorial arrangement of treatment effects (temperature, live *M. leprae*, dead *M. leprae* and non-infected controls). Post hoc analyses were conducted with pairwise-t-test comparisons of least square means and all probabilities were considered significant at ( $p \leq 0.05$ ).

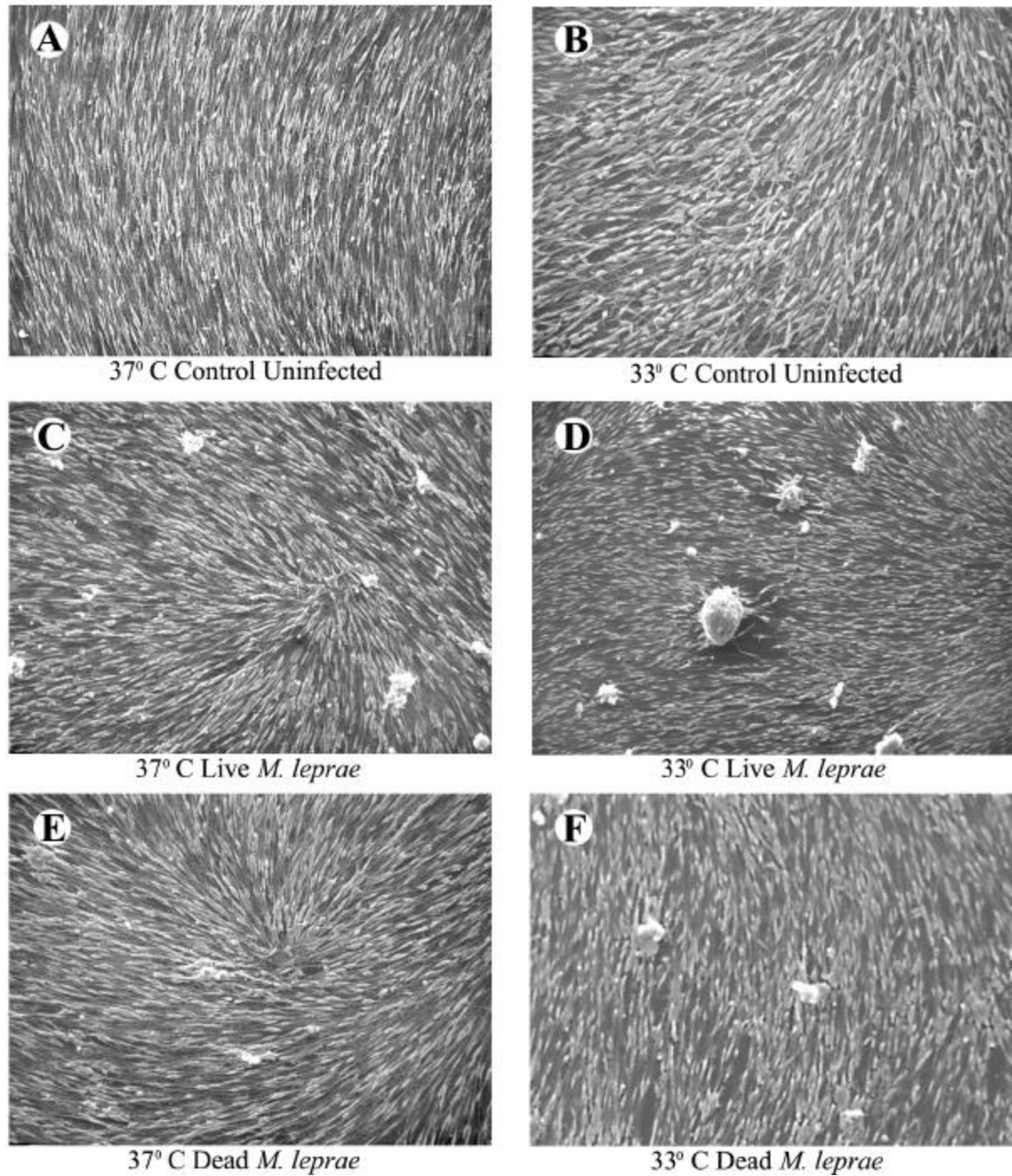
### 2.2.3. Results

Schwann cells cultivated at 33<sup>0</sup> C appeared to survive and function in a manner similar to those cultivated at 37<sup>0</sup> C. For example, these cells formed typical swirled monolayers (**Fig. 2.1.B**), appeared to maintain their viability and survived for several weeks in culture. In addition, the level of expression of specific genes which encode typical Schwann cell markers was comparable to that at 37<sup>0</sup> C at 3 d post-temperature change (**Fig. 2.2.**).

When Schwann cells were incubated at 33<sup>0</sup> C for as long as 15 d prior to being seeded onto axons of embryonic neurons, they were able to attach, align and proliferate along axons in a manner comparable to those at 37<sup>0</sup> C (**Fig. 2.3.**). After these co-cultures were maintained in medium containing L-ascorbic acid, the Schwann cells myelinated some of the axons and appeared to be able to maintain myelin sheaths for at least one month at 33<sup>0</sup> C (**Fig. 2.4.**). These results were similar to those observed at 37<sup>0</sup> C (data not shown). Myelinated and non-myelin producing Schwann cells were observed in all co-cultures at both temperatures.

Myelin sheath architecture as well as Schwann cell surface membranes, mitochondria, organelles and nuclear membranes were similar in co-cultures developed at both temperatures (**Fig. 2.4.**). Schwann cell/axonal membrane interfaces were contiguous and comparable at both temperatures.

Axons and neural cell bodies at both temperatures exhibited no apparent differences. Surface membranes, organelles, mitochondria and microtubules were comparable between temperatures. Established myelinated co-cultures originally produced at 37<sup>0</sup> C and moved to 33<sup>0</sup> C for up to one month also failed to develop any apparent alterations in Schwann cell or axonal appearance.



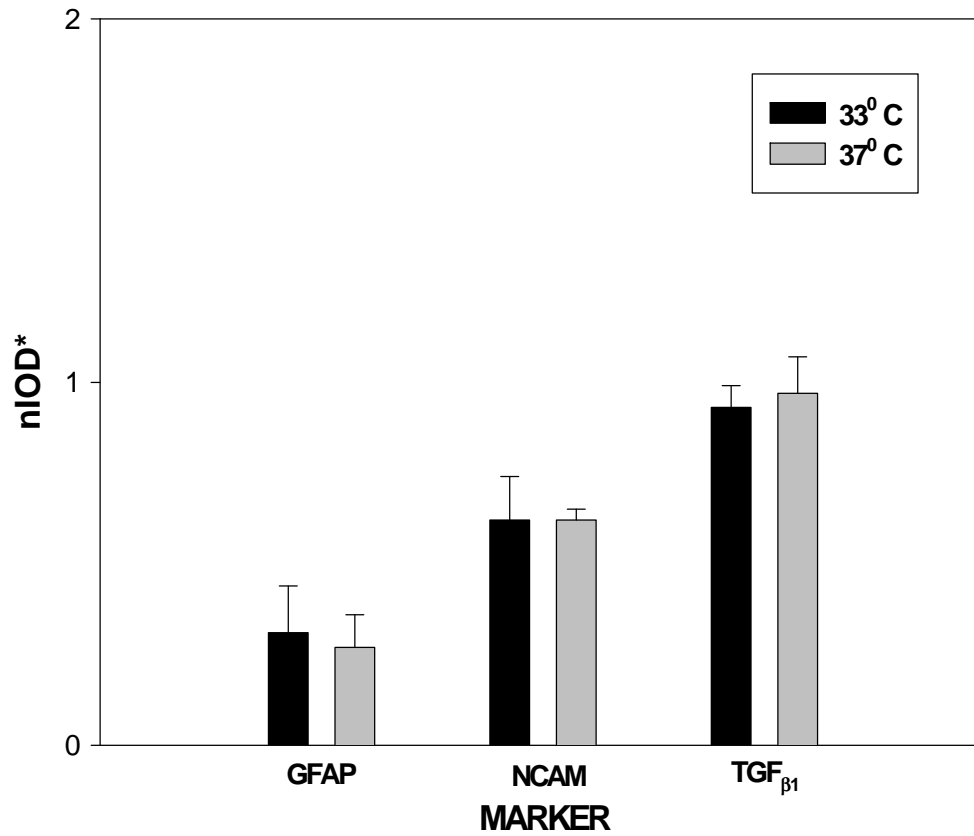
**Figure 2.1.** Scanning electron micrographs of Schwann cell monolayers 12 d post-infection. Purified primary rat Schwann cell monolayers were exposed to viable (Live) or irradiated (Dead) *M. leprae* harvested from the footpads of nude mice for 48 h. The monolayers were maintained at either 33° C or 37° C for up to 21 d post-infection. Micrographs are shown at approximately 60x final magnification

Purified *M. leprae* preparations harvested from foot pads of *nu/nu* mice and used for infection experiments had a mean BACTEC growth index (GI)  $152 \pm 8$  indicating that they contained highly viable *M. leprae* (Franzblau, S.G. 1989). Exposure of Schwann cell monolayers to these bacteria at an MOI = 100 for 48 h, resulted in a mean phagocytic index of  $20 \pm 5$  *M. leprae* per Schwann cell and > 99% infection (**Fig. 2.5.**). Buddemeyer radiorespirometry results of these cultures demonstrated that *M. leprae* within Schwann cells retained 56% of their initial viability up to 21 days post-infection in Schwann cells maintained at 33<sup>0</sup> C compared to only 3.5% at 37<sup>0</sup> C (**Fig. 2.6.**).

Infected Schwann cells at 33<sup>0</sup> C appeared to retract from the surface of the flask; resulting in the loss of the typical ‘swirled’ monolayer appearance (**Fig. 2.1. B, C**). The majority of monolayers containing cell aggregates were observed in wells containing Schwann cells infected with metabolically active *M. leprae* at 33<sup>0</sup> C (**Table 2.3.**). Schwann cells in these cultures appeared to retract from the monolayers. None of the live infected Schwann cells at 37<sup>0</sup> C exhibited this altered monolayer morphology. A much lower number of wells were observed to contain clumps in cultures infected with irradiated *M. leprae*.

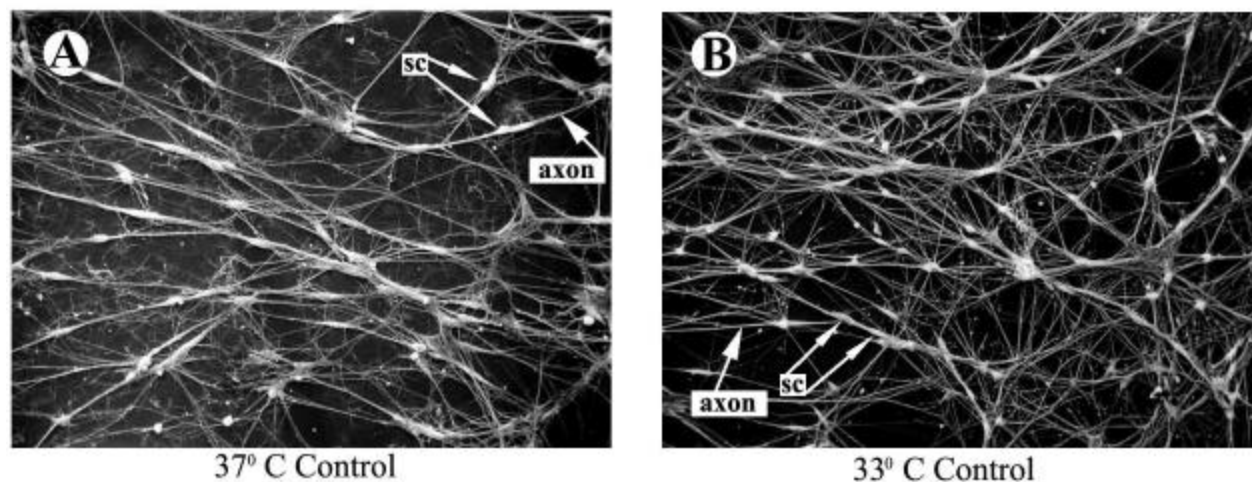
Schwann cell monolayers were analyzed 12 d post-infection by both scanning electron microscopy (SEM) and transmission electron microscopy (TEM) microscopy. SEM analysis demonstrated that while the infected monolayers had similar amounts of tissue debris (**Fig. 2.1., 2.7.**), only the cultures with metabolically active *M. leprae* exhibited altered Schwann cell morphology. SEM revealed Schwann cells in clumps were tightly packed together, mixed with some tissue debris and typically surrounded by a cleared area from which the monolayer had appeared to retract (**Fig 2.8.**).





\* normalized integrated optical density (nIOD)  
determined by sample IOD /housekeeping IOD

**Figure 2.2.** Comparison of Schwann cell gene expression 3 d after temperature change. RT-PCR analysis of RNA collected from Schwann cells incubated at 33<sup>0</sup> C and 37<sup>0</sup> C. GFAP = glial fibrillary acidic protein  
NCAM = neural cell adhesion molecule  
TGFβ1 = transforming growth factor-beta 1

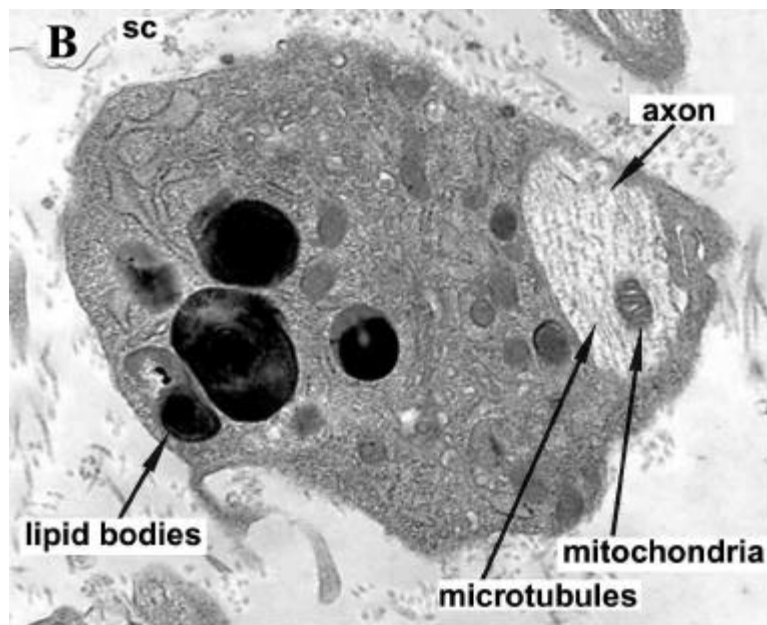
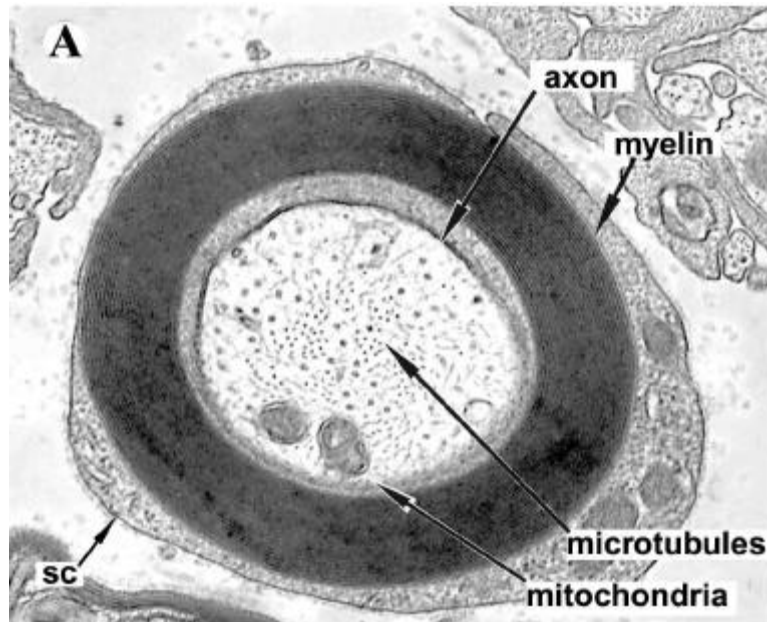


**Figure 2.3.** Scanning electron micrographs of purified primary Schwann cells seeded onto purified neurons and then incubated for 24 h at 33° C and 37° C. Micrographs are shown at approximately 150x final magnification.

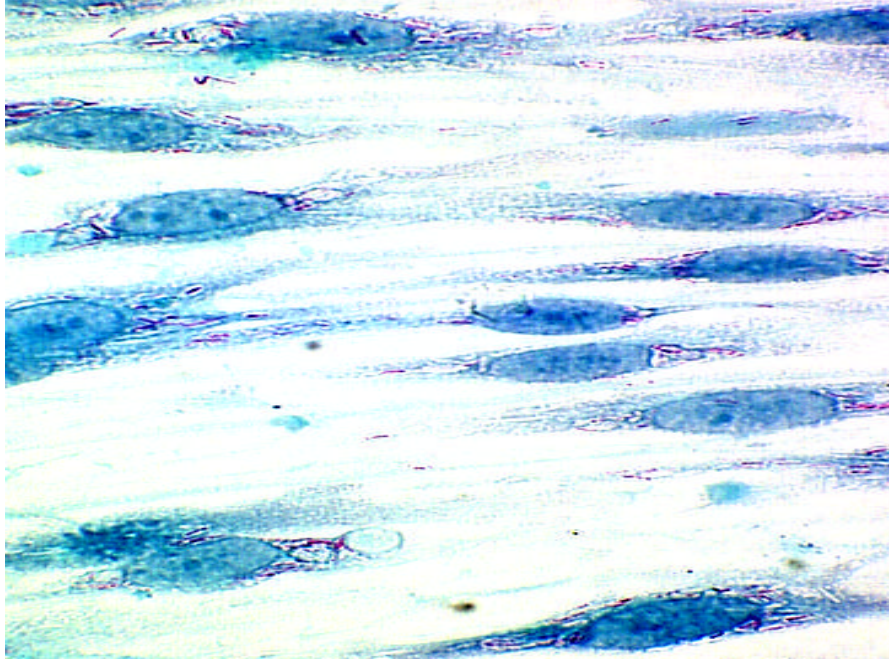
TEM analysis of infected Schwann cell cultures revealed that clumps or portions of retracted monolayers contained Schwann cells with notable microscopic Schwann cell/Schwann cell interaction alterations (**Fig 2.9.**). The tightly packed infected Schwann cells within the retracted areas exhibited more contact with neighboring cells than was observed in other unaffected monolayers. Individual Schwann cell surface membranes remained visibly distinguishable, indicating that the mass of Schwann cells was not syncytial. Additionally, the center of the clumps of Schwann cells revealed unidentified amorphous material.

## 2.4 . Discussion

Despite advances in neurobiology and neuroimmunology, the mechanisms underlying nerve damage in leprosy have not been fully elucidated but appear to involve direct effects of *M. leprae* infection and an aggressive immune response to *M. leprae* or its antigens (Spierings, E. *et al*, 2001). One reason that these events still remain obscure is the lack of a good *in vivo* or *in vitro* model that sustains *M. leprae* viability and closely mimics *in vivo* disease conditions.

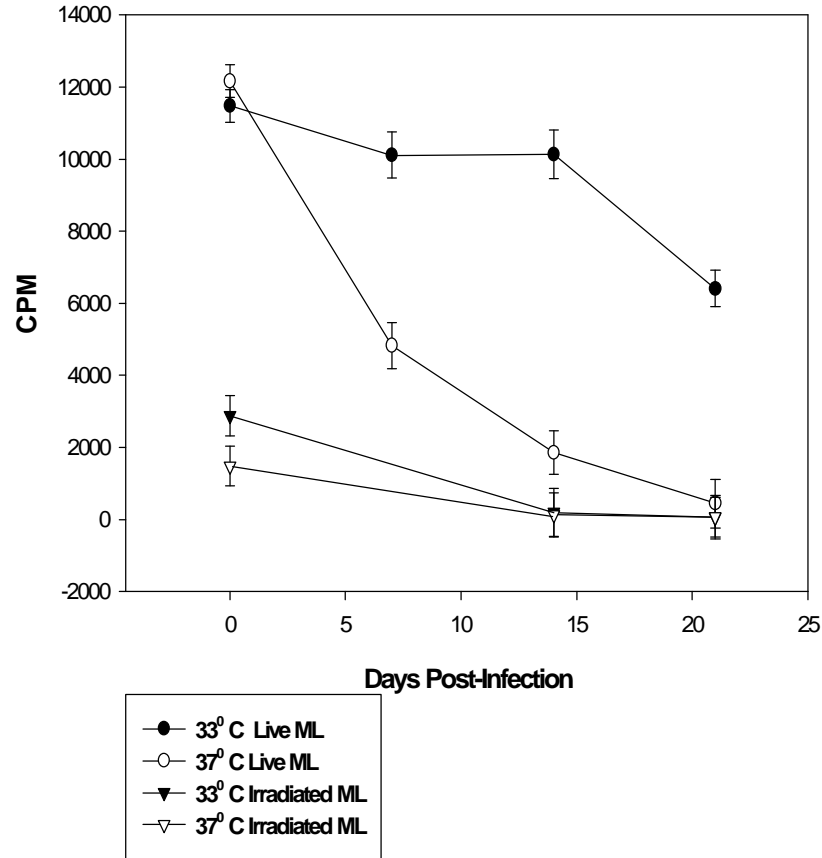


**Figure 2.4.** Transmission electron micrograph of myelinated (A) and non-myelin-producing Schwann cells (B) within a Schwann cell/neuron co-culture developed and maintained at 33<sup>0</sup> C. Schwann cells (SC) were seeded onto purified neurons at 33<sup>0</sup> C, allowed to proliferate for two weeks and then stimulated to produce myelin.



**Figure 2.5.** Thick section acid fast micrograph of primary Schwann cells infected 48 h post-inoculation with live *M. leprae* at 33<sup>0</sup> C. Sections were viewed under oil immersion (1000x) using light microscopy. Purified primary Schwann cells were exposed to viable *M. leprae* freshly harvested from the footpads of nude mice for 48 h and then fixed and stained as described by Luderschmidt, C. (1987).

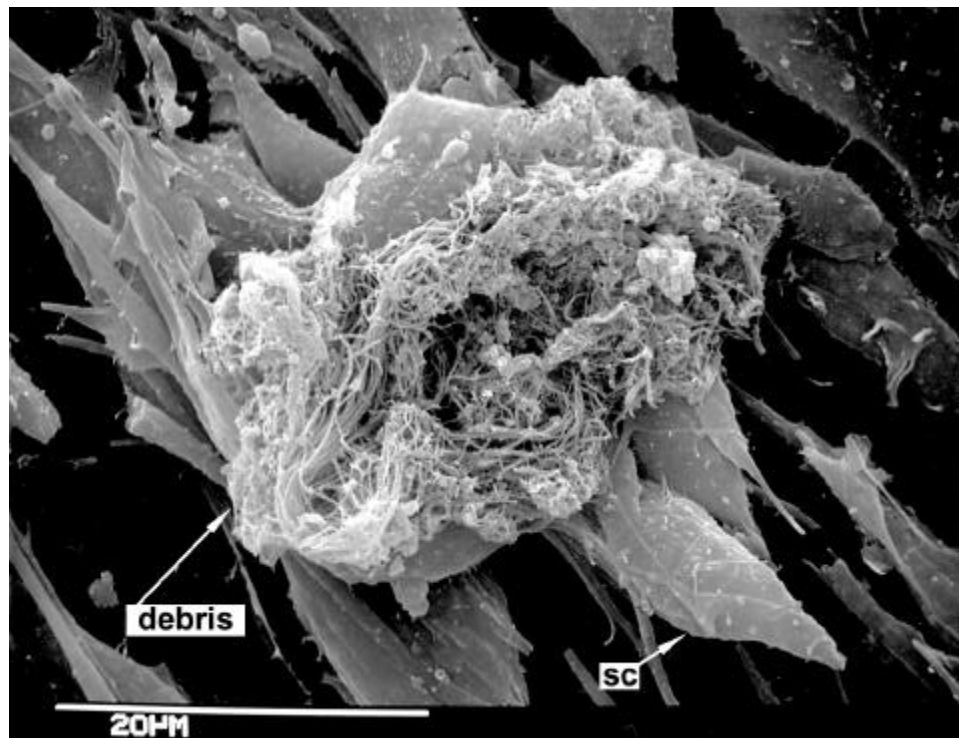
Histopathological and molecular evidence have demonstrated that the Schwann cell serves not only as the natural host for the growth and replication of *M. leprae* in the peripheral nerve, but suggests that it protects this obligate intracellular organism from immune responses and exposure to therapeutic agents due to the blood-nerve barrier. Recently Rambukkana *et al* (1997) defined the mechanism of neural targeting of *M. leprae* to the Schwann cell via the lamina  $\alpha$ -2 chain located on the surface of the basal lamina of the Schwann cell within the Schwann cell-axon unit. Ng *et al* (2000) suggested that the *M. leprae* invasion of the Schwann cell is not a bacterial-driven phenomenon but rather a passive invasion process in which the basal lamina and Schwann cell play the major role in response to the cell wall components, such as, phenolic glycolipid-1 (PGL-1).



**Figure 2.6.** Buddemeyer radiorespirometric analysis of metabolic activity of *M leprae* (ML) within Schwann cells *in vitro*. Purified primary Schwann cells containing viable *M. leprae* harvested from the footpads of *nu/nu* mice were incubated at either 33° C or 37° C for up to 21 d post-infection. For each time point, Schwann cells were lysed and the bacteria were analyzed for metabolic activity (radiolabelled CO<sub>2</sub> release) for 7 d using the Buddemeyer radiorespirometric system (Franzblau, S.G., 1989).

**Table 2.3.** Schwann cell aggregate formations in purified primary rat Schwann cell monolayers infected with *M. leprae*.

Schwann Cells	# Aggregates	Wells with aggregates
33 <sup>0</sup> C Control	0	0/19
33 <sup>0</sup> C Live <i>M. leprae</i>	79	15/19
33 <sup>0</sup> C Dead <i>M. leprae</i>	12	8/19
37 <sup>0</sup> C Control	0	0/19
37 <sup>0</sup> C Live <i>M. leprae</i>	0	0/19
37 <sup>0</sup> C Dead <i>M. leprae</i>	5	3/19



**Figure 2.7.** Scanning electron micrograph taken at high magnification of debris from *M. leprae* preparation that adhered to the primary Schwann cell monolayer.

Several investigators have shown that once attached to the Schwann cell surface bacteria are rapidly ingested (Band, A.H. *et al*, 1986a & 1986b; Mukherjee, R., 1986; Steinhoff, U. *et al*, 1989). However, once *M. leprae* gains intracellular access, there are conflicting reports on the effects of infection on Schwann cell survival and functional capabilities (Steinhoff, U. *et al*, 1991; Mistry, Y. *et al*, 1992; Mukherjee, R. and Antia, N.H., 1985; Singh, N. *et al*, 1998). In addition, no comprehensive study has been conducted to determine the fate of *M. leprae* within cultured Schwann cells.

Mukherjee *et al* (1981) showed that infection leads to the inability of infected Schwann cells in co-culture with neurons to synthesize DNA and that *M. leprae* infection leads to the inability of Schwann cells to associate with axons of neurons in nerve explant cultures. Recently Ng, *et al* (2000) suggested that the retention of dead bacilli within nerve fibers can continuously activate Schwann cells and possibly dysregulate nerve functions. In addition, they report unpublished observations that both dead *M. leprae* and PGL-1 have the capacity to induce nerve damage upon binding to nerve fibers suggesting a role of PGL-1 in nerve pathogenesis in leprosy. Therefore, the direct effects of *M. leprae* infection on Schwann cells and Schwann cell/neuron units which may potentially lead to the neuropathology observed in leprosy still remain unclear.

A potential reason for the various conflicting reports on the proposed causes of neuropathy is the lack of a standardized model of infection that effectively models the active disease state. Bacteria were obtained from a variety of sources including; human biopsies (Mukherjee, R. *et al*, 1981; Shetty, V.P. *et al*, 1994; Suneetha, L.M. *et al*, 1998), mouse foot pads (Saito, H. *et al*, 1986; Suneetha, L.M. *et al*, 1998), and armadillo tissues (Steinhoff, U. *et al*, 1989; Birdi, T.J. *et al*, 1997; Ng, V. *et al*, 2000; Rambukkana, A. *et al*, 1997; Shimoji, Y. *et*

*al*, 1999). In addition, the viability of these preparations was not determined using the gold standard= mouse foot pad inoculation method (Sheppard, C.C. *et al*, 1960) or other methods such as radiorespirometry developed for determining *M. leprae* viability (Franzblau, S.G., 1989).

Truman and Krahenbuhl (2001) have recently demonstrated that the viability of *M. leprae* harvested from different sources varies greatly and concluded that bacteria serially passaged and harvested from the foot pads of BALBc *nu/nu* mice were the most viable.

Previous studies have also used *M. leprae* that were frozen and thawed (Steinhoff, U. *et al*, 1989; Singh, N. *et al*, 1996; Maeda, M. and Narita, M., 1987; Singh, N. *et al*, 1997) or held at various temperatures including 37<sup>0</sup> C prior to and during Schwann cell infection experiments (Rambukkana, A. *et al*, 1997; Ng, V. *et al*, 2000; Mukherjee, R. *et al*, 1981). Truman and Krahenbuhl (2001) have recently shown that several of these standard laboratory practices dramatically reduce *M. leprae* viability. Freezing *M. leprae* at -80<sup>0</sup> C then thawing reduced the viability of these bacteria by 99.9% when compared to that of freshly harvested *M. leprae*. Storage of freshly harvested *M. leprae* in 7H12 medium at 37<sup>0</sup> C reduced its viability by greater than 98% in 48 h. In comparison, storage of *M. leprae* at 4<sup>0</sup> C or 33<sup>0</sup> C in the same medium did not reduce the viability of *M. leprae* over this same time period (Truman and Krahenbuhl, J.L., 2001). Therefore, bacteria used for the majority of previous studies to investigate *M. leprae* Schwann cell interactions were either dead prior to infection or died shortly after infection.

Since the viability of a bacterial preparation may have a major influence on experimental outcome, the results of previous studies may not correctly reflect the effects of viable *M. leprae* on Schwann cells (Truman and Krahenbuhl, J.L., 2001; Mistry, Y. *et al*, 1992). Although dead bacilli have been shown to elicit significant and specific effects on Schwann cells

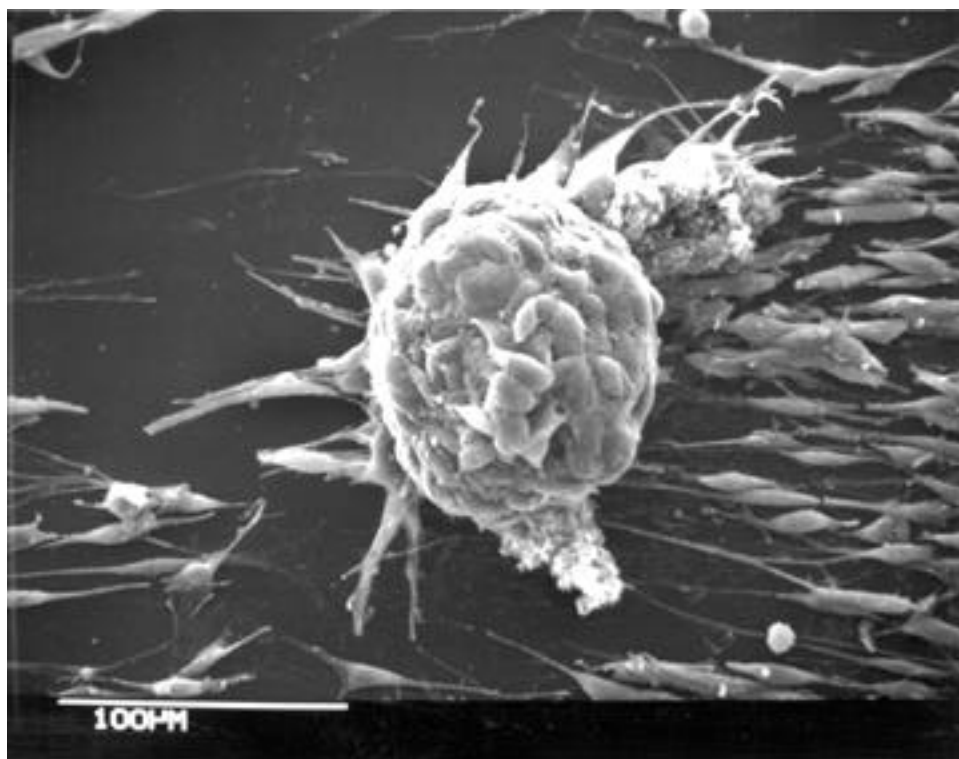


that may be important in the overall nerve destruction process, it is imperative that experimentation purporting to mirror the live disease infection state use viable bacteria.

It has also been well established that *M. leprae* infection causes a systemic infection and bacteria grow to high numbers when injected into armadillos, whose core body temperature is 33<sup>0</sup> C (Storrs, E.E., 1971; Kirscheimer, W.F. *et al*, 1972; Walsh, G. *et al*, 1986; Truman and Sanchez). In addition, *M. leprae* are routinely found in human leprosy patients in the cooler regions of the body (30-34<sup>0</sup> C), such as, the skin, peripheral nerves, and the anterior segment structures of the eye (Adams, L.B. and Krahenbuhl, J.L., 1996). It is in these locations that *M. leprae* in lepromatous patients grow to very high numbers and exhibit their greatest detrimental effects. Therefore, this information also suggests that *M. leprae* have greater viability at temperatures that are lower than core body temperature (37<sup>0</sup> C). It also indicates that *in vitro* infection studies should be conducted at 33<sup>0</sup> C to retain maximal viability of this pathogen as well as to mimic the temperature preference demonstrated by *in vivo* sites of infection.

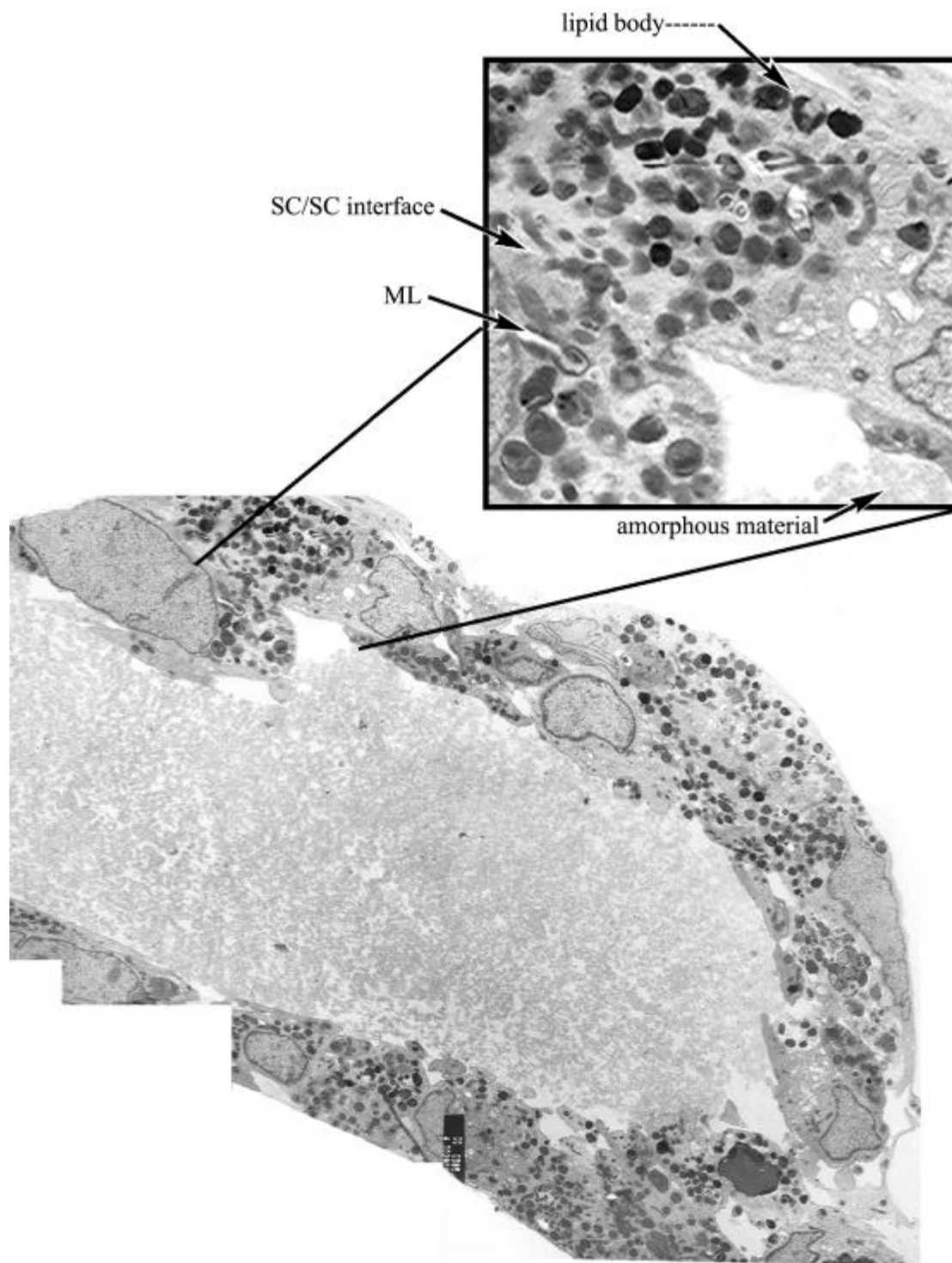
Schwann cells and neurons used in previous reports were obtained from a variety of sources, such as: human tissues (Ng, V. *et al*, 2000; Suneetha, L.M.), mouse tissues (Steinhoff, U. *et al*, 1989; Saito, H. *et al*, 1986; Birdi, T.J. *et al*, 1997), and rat tissues (Rambukkana, A. *et al*, 1997; Ng, V. *et al*, 2000; Shimoji, Y. *et al*, 1999; Singh, N. *et al*, 1997). In addition, appropriate specific assays for fibroblast contamination were not used to define Schwann cell purity in most of these studies; therefore, the identity of the cells described may not have been valid.

Transformed glial cell lines such as 33B (Band, A.H. *et al*, 1986) and S16 (Sasagasako, N. *et al*, 1999) are also commonly used in Schwann cell research. These transformed cell lines are easily maintained on tissue culture medium. Although transformed cell lines have been used



**Figure 2.8.** Scanning electron micrograph a cell aggregate of Schwann cells infected with live *M. leprae* at 33<sup>0</sup> C. Purified primary rat Schwann cell monolayers were exposed to viable or irradiated *M. leprae* harvested from the footpads of nude mice and then maintained at 33<sup>0</sup> C for up to 21 d post-infection.

to study *M. leprae* adherence mechanisms and phagocytic index (Band, A.H. *et al*, 1986; Mistry, Y. *et al*, 1991; Maeda, M. and Narita, M., 1987), their usefulness for investigating long-term intracellular effects of *M. leprae* on the Schwann cell or long term viability of *M. leprae* within the Schwann cell is severely limited. The transformed cell lines typically have an extremely rapid growth rate (24 h) and, since there is no evidence of *M. leprae* growth within these cells, infection is in effect diluted every 24 h. In addition, these cells have lost typical Schwann cell functional characteristics, such as the ability to associate and interact with neurons, as a function of the dedifferentiation process that accompanies transformation. These traits render transformed cells unsuitable for studies to define the effects of infection on Schwann cells or Schwann cell/neuron interactions.



**Figure 2.9.** A collage of transmission electron micrographs of a primary Schwann cell aggregate that formed Schwann cell monolayers infected with live *M. leprae* and maintained at 33<sup>0</sup> C. The collage original is shown at approximately 7,200x final magnification with an inset of 18, 000x.

Since leprosy is a disease of humans, it is logical that human Schwann cells and neurons should be the cells of choice for these studies. However, only a limited number of studies have been done using human Schwann cells (Ng, V. *et al*, 2000; Spierings, E. *et al*, 2001). One reason for this is that they are difficult to obtain since they must be harvested from donated organs, amputated limbs or excised nerve tissue. This requires access to specific medical facilities and trained medical personnel. In addition, since all donors vary in age, Schwann cells obtained from these sources will vary in condition and, potentially, functional capabilities from one harvest to another. Efficient myelination of axons in human Schwann cell/neuron co-cultures has not been achieved (Personal communication Dr. Patrick Wood, Miami Project to Cure Paralysis, University of Miami Medical School, Miami, FL). Therefore, there have been only a limited number of studies using human Schwann cell cultures to define the effects of *M. leprae* infection on these cells (Ng, V. *et al*, 2000; Suneetha, L.M.).

Purified mouse or rat Schwann cells, and neurons have been used successfully to develop age-synchronized cultures, for the efficient development of myelinated Schwann cell/neuron co-cultures (Kleitman, N., 1996). These cultures have been used to define many aspects of basic Schwann cell biology, Schwann cell/neuron interactions and various aspects of *M. leprae*/Schwann cell interactions (Mukherjee, R., 1980a; Mukherjee, R., 1980b; Mukherjee, R. and Antia, N.H. 1985). Neonatal and embryonic rats, used as sources of these cells, can be obtained from time-pregnant females which can be readily purchased from commercial sources or can be obtained from an established laboratory rat colony. Therefore, these cells are a logical choice as an *in vitro* model for studying *M. leprae*/Schwann cells interactions. However, no comprehensive studies have been conducted to determine the effect of 33<sup>0</sup> C temperature on *M.*

*leprae* viability within these cells or the effect on Schwann cell survival or functional capabilities.

Therefore, to establish an improved model for studying *M. leprae*/Schwann cell interactions, a comprehensive study was conducted to determine the effects of 33<sup>0</sup> C on Schwann cell survival, replication and differentiation into mature myelinating and non-myelin producing Schwann cells in the presence of neurons. The findings of this study demonstrated that Schwann cells showed typical "swirled" monolayer morphology, appeared to survive as long and had comparable levels of specific RNA transcripts for GFAP, NCAM and TGFβ1 as those cells maintained at 37<sup>0</sup> C.

When Schwann cells were seeded onto neurons at 33<sup>0</sup> C, they aligned and associated with the axons, proliferated along the axons and produced myelin upon stimulation similar to parallel cultures incubated at 37<sup>0</sup> C. Both myelinated and nonmyelin-producing Schwann cells were observed in the co-cultures at both temperatures.

The incubation temperature had no apparent effect on Schwann cell or axonal structure or function when analyzed by electron microscopy. No aberrations or apparent differences were observed between Schwann cell or axon membrane interfaces, membrane integrity or Schwann cell organelles at either temperature. Myelin sheath formation and architecture were comparable and seemed to vary, as expected, with the diameter of the axon ensheathed by the Schwann cell. Therefore, based on the parameters studied, it appeared that rat Schwann cells are as functionally competent at 33<sup>0</sup> C as their counterparts held at 37<sup>0</sup> C.

Data from the present study also support the conclusion that *M. leprae* preparations harvested from the *nu/nu* foot pads reproducibly demonstrated highly viable *M. leprae* as these preparations contained BACTEC GIs > 100. Franzblau, S.G. (1989) previously demonstrated

that bacterial preparations containing a GI  $\geq 100$  yielded highly viable *M. leprae* as determined by the mouse foot pad inoculation assay.

In addition, *M. leprae* retained significantly greater metabolic activity within Schwann cells maintained at 33<sup>0</sup> C than those maintained at 37<sup>0</sup> C, indicating that 33<sup>0</sup> C supports short-term viability of *M. leprae* in cultured Schwann cells. Truman and Krahenbuhl (2001) demonstrated that *M. leprae* in axenic cultures maintained at 33<sup>0</sup> C lost on average 20% of their metabolic activity within the first week after harvest from an infected nude mouse footpad and retained 10% of their metabolic activity after three weeks. They also found that regardless of the incubation temperature, most *M. leprae* suspensions retained less than 10% of their original metabolic activity after three weeks and grew poorly when inoculated into a mouse foot pad.

Truman and Krahenbuhl (2001) also demonstrated that *M. leprae*, held in axenic medium at 37<sup>0</sup> C, rapidly lost all detectable metabolic activity within only a few days. Results from the present study demonstrated that when held within Schwann cells at 37<sup>0</sup> C, *M. leprae* was able to retain viability two weeks longer than bacteria in axenic medium, though significantly less than parallel cultures infected with *M. leprae* held at 33<sup>0</sup> C. Since *M. leprae* preferentially infects the Schwann cell and is an obligate intracellular parasite of this cell type *in vivo*, it is, therefore, logical to assume that *M. leprae* retains metabolic activity for longer periods within Schwann cells, compared to axenic medium. Our data demonstrated that *M. leprae* retains significantly more metabolic activity for at least three weeks within cultured primary rat Schwann cells held at 33<sup>0</sup> C than at 37<sup>0</sup> C. Therefore, these data strongly suggest that experiments to determine the effects of viable *M. leprae* on Schwann cells should be conducted at 33<sup>0</sup> C.

When infected with live *M. leprae* and held at 33<sup>0</sup> C, Schwann cell monolayers exhibited alterations in monolayer morphology within 4-6 d post-infection. Large clumps of Schwann

cells that retracted from the monolayer were shown by TEM to consist of tightly packed infected Schwann cells surrounding unidentified amorphous material. This amorphous material could possibly be dead, degenerated Schwann cells or mouse tissue debris that remained from the *M. leprae* harvest suspension. Although mouse tissue was present in both live and irradiated infected cultures at both temperatures, cells only retracted from the monolayer in cultures held at 33<sup>0</sup> C and primarily those that were infected with metabolically active *M. leprae*. These results indicate that Schwann cells at 33<sup>0</sup> C respond in a differential manner to metabolically active *M. leprae*.

Data presented within this study indicate that infected cultures maintained at 33<sup>0</sup> C retain higher metabolic activity within the bacteria; however, definitive analyses have not been performed to rule out the possibility that the Schwann cells are also different due to the temperature change. More extensive gene transcription analyses, such as Differential Display analysis or cDNA array analysis, would provide broader methods to investigate the effects of temperature on the Schwann cells at the molecular level. The direct correlation of metabolic activity to Schwann cell morphological response within this study indicates that live *M. leprae* infection at 33<sup>0</sup> C does affect the Schwann cell, though it cannot be said that the lowered temperature does not also have a role in the response of the Schwann cell to live infection.

Adams *et al* (2000) demonstrated that *M. leprae* can be effectively killed by 10<sup>6</sup> rad of  $\gamma$ -irradiation, as shown by loss of metabolic activity and growth potential when inoculated into the footpad of a nude mouse. However, metabolic activity as measured by the BACTEC 460 respirometry system indicated that loss of metabolic activity, although rapid (within days), was not immediate. Our data also support these observations. Irradiated *M. leprae* used immediately

after irradiation to infect Schwann cells at 33<sup>0</sup> C retained a small amount of metabolic activity for a brief period of time.

TEM analysis also demonstrated amorphous material within the infected Schwann cell aggregates. The amorphous material could be degenerating Schwann cells or remnant mouse footpad tissue from the *M. leprae* preparation that adhered to monolayer. Debris was present on the surface of the Schwann cell monolayers in all cultures exposed to bacteria; however, the differential response occurred only within cultures containing metabolically active *M. leprae*. It is possible that additional bacteria associated with tissue debris that remained adherent after the initial wash (48 h) could have provided additional exposure to *M. leprae* resulting in higher phagocytic indices of the surrounding Schwann cells. Further analysis would be required to determine if Schwann cells within the aggregates do contain more *M. leprae* than Schwann cells that do not aggregate.

The response of Schwann cells to infection by metabolically active *M. leprae* at 33<sup>0</sup> C may be indicative of altered Schwann cell/Schwann cell interactions. When this was analyzed by TEM, it was observed that tightly packed cells had more cell surface membrane contact with neighboring cells than cells from the other experimental sets. Therefore, it is conceivable that infection altered adhesion molecules on the surface of the Schwann cell, which resulted in an increased binding of these cells to each other.

The direct effects of *M. leprae* on the Schwann cell gene expression of adhesion molecules at 33<sup>0</sup> C are not known. Adhesion molecule alteration could alter the relationship of Schwann cells to other Schwann cells and axons, as well as, immune cells patrolling the tissue or responding to infection. Adhesion molecules have been shown to play a critical role in the proper relationship between the axon and Schwann cell which is a mandatory for the production



and maintenance of myelin (Fu, S.Y. and Goron, T. 1997). Any modification of this relationship could alter the Schwann cell's ability to produce and maintain myelin; thereby, potentially inducing myelin damage or demyelination.

Adhesion molecules also have a critical role in the management of the regenerative response of the Schwann cell to damage within the peripheral nerve. The expression of Schwann cell adhesion molecules such as NCAM, N-cadherin and L1 have been shown to have direct involvement in neurite outgrowth promotion, the Schwann cell/axon relationship and myelination (Nieke, J. and Schachner, M. 1985; Martini, R. and Schachner, M., 1988; Cifuentes-Diaz, C. *et al*, 1994). The regulation of these adhesive glycoproteins on the surface of both the neuron and Schwann cell may determine the extent of reinnervation that occurs following peripheral nerve damage (Fu, S.Y. and Goron, T. 1997).

Therefore further studies are necessary to determine the gene expression and potential functional response of Schwann cells infected with metabolically active *M. leprae* at 33<sup>0</sup> C. Specifically, investigations need to be performed to evaluate the expression of adhesion molecules that are critical to the regenerative role of the Schwann cell as well as the functional aspects of the relationship of the metabolically active *M. leprae*-infected Schwann cells and axons and resulting axonally-dependent activities such as the production and maintenance of myelin.

In summary, the results of this study clearly demonstrated that Schwann cells, cultured at 33<sup>0</sup> C, appear to survive and function in a comparable fashion to those incubated at 37<sup>0</sup> C and that *M. leprae*'s metabolic activity is maintained longer within Schwann cells at 33<sup>0</sup> C than at 37<sup>0</sup> C. Therefore, this study concludes that an improved, standardized model for studying *M. leprae*/Schwann cell and Schwann cell/neuron interactions has been developed. This model uses

highly viable, nude mouse foot pad-derived *M. leprae* and purified primary Schwann cells and Schwann cell/neuron co-cultures, and maintains these cultures at 33<sup>0</sup> C prior to and during infection studies.

It is anticipated that this model will be useful for identifying the additional effects of *M. leprae* infection on Schwann cells and Schwann cell/neuron interactions. A better understanding of the direct effects of viable *M. leprae* infection of the Schwann cell will potentially grant a greater understanding of the factors that contribute to the neuropathy observed in leprosy infection. In addition, the use of a standardized model will also be critical for defining the role that specific immune factors, such as, cytokines and specific immune cells have on infection.

### CHAPTER 3.

#### EFFECTS OF *MYCOBACTERIUM LEPRAE* INFECTION ON SCHWANN CELL ADHESION MOLECULE GENE EXPRESSION

##### 3.1. Introduction

*Mycobacterium leprae*, the causative agent of leprosy, is an obligate intracellular parasite that has been shown to specifically target the Schwann cell of the peripheral nerve system resulting in demyelination and axonotrophy (Rambukkana, A., 1997; Shetty, V.P. *et al*, 1994). While the mechanisms of neuropathology are not well understood, current hypotheses propose that nerve damage is a direct result of Schwann cell infection and immune reaction within the sensitive nerve tissues (Spierings, E. *et al*, 2001). It is, however, conceivable that the intracellular presence of the bacilli could alter Schwann cell gene expression and thereby alter the Schwann cell's functional relationship with an axon, myelin production or maintenance and potential regenerative capability.

Previous experiments in our laboratory using a new *in vitro* model to study *M. leprae*/Schwann cell interactions have demonstrated that primary rat Schwann cells infected with metabolically active *M. leprae* for at least 4 days at 33<sup>0</sup> C exhibited altered morphology. Schwann cells appeared to retract from the monolayer into large clumps, resulting in the loss of the typical swirled Schwann cell monolayer morphology (**Section 2. 3.**). In addition, it appeared that Schwann cell/Schwann cell binding was altered in these cultures. These effects were not observed in metabolically active *M. leprae*-infected Schwann cells held at 37<sup>0</sup> C which indicated that the results were related to the viability of the *M. leprae* within these cultures.

The alteration of Schwann cell/Schwann cell interactions in the above experiments suggests that the expression of adhesion molecules may be altered by infection. These molecules have critical roles in regulating Schwann cell mediated nerve regeneration as well as Schwann

cell/neuron interactions necessary for myelin production and maintenance by the differentiated myelinating Schwann cell. During the regenerative process, Schwann cells dedifferentiate into a state similar to the premyelin phenotype, proliferate within the traumatized region and then re-associate with axons in order to repair and re-myelinate the damaged area (Fu, S.Y. and Goron, T., 1997). Therefore, Schwann cells function to stimulate and mediate nerve tissue regeneration following nerve trauma or injury and to maintain myelinated axons in peripheral nerve neurons (Bixby, J.L. *et al*, 1988). Alteration of cell surface adhesion molecules (CAMs) or molecules which regulate the expression of these molecules may have a great impact on the Schwann cell's ability to perform these functions.

Histopathological evidence obtained from leprosy patient nerve biopsies has demonstrated that neuropathy such as nerve fiber degeneration, increased numbers of small myelinated fibers as a possible consequence of axonal atrophy and loss of unmyelinated axons occurs at various stages of *M. leprae* infection (Shetty, V.P. *et al*, 1988). Therefore, it is plausible that one of the direct effects of *M. leprae* infection on the Schwann cell is the alteration of CAM molecule gene expression. This may result in an alteration of the type or the amount of CAMs that are expressed on the surface of Schwann cells at very critical stages of Schwann cell differentiation.

The regenerative process of Schwann cells is regulated by or involves numerous factors including glial fibrillary acidic protein (GFAP) and transforming growth factor beta-1 (TGF $\beta$ -1) (Einheber, S. *et al*, 1995). GFAP is the major protein constituent of glial intermediate filaments (Chen, W.J. *et al*, 1994) and has also been shown to have an important role in Schwann cell/neuron interactions. TGF $\beta$ -1, an important cell mediator of response to trauma, has been shown to modulate Schwann cell phenotype as well as the expression of other key molecules (Mews, M.

and Meyer, M., 1993; Guenard, V. *et al*, 1995). TGF $\beta$ 1 transcript levels have been shown to dramatically increase after nerve injury (Rogister, B. *et al*, 1993; Scherer, S.S. *et al*, 1993), increase NCAM expression, and be chemotactic for macrophages. TGF $\beta$ 1 also inhibits myelination in Schwann cell/neuron co-cultures and can lead to defects in the formation of the basal lamina of the Schwann cell (Einheber, S. *et al*, 1995).

Various CAMs such as L1, neural cell adhesion molecule (NCAM), intercellular adhesion molecule (ICAM) and N-cadherin have also been shown to have pivotal roles in the regenerative process of Schwann cells (Nieke, J. and Schachner, M.,1985; Martini, R. and Schachner, M.,1988; Cifuentes-Diaz, C. *et al*, 1994). These integrin-class extracellular matrix receptors have been shown to have direct involvement in neurite outgrowth promotion as well as in the critical relationship between the Schwann cell and the ensheathed axon which is required for appropriate formation and maintenance of the basal lamina and myelin. L1 and NCAM have also been shown to have altered expression which correlated with regenerative activity following nerve transection (Tacke, R. and Martini, R., 1990). The regulation of these adhesive glycoproteins on the surface of both the neuron and Schwann cell may determine the extent of reinnervation that occurs following peripheral nerve damage.

In this report, we demonstrate the usefulness of mitogen-expanded Schwann cells in the improved infection model. In addition, total RNA from infected Schwann cells was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) to determine the effects of infection on various adhesion molecule transcripts known to have important roles in Schwann cell/axon interaction and the regenerative response. Findings indicated that significant gene expression alteration did occur as a result of infection with metabolically active *M. leprae*.

## **3.2. Materials and Methods**

### **3.2.1. Bacteria**

*M. leprae* Thai-53 were purified from the hind foot pads of nude BALBc (*nu/nu*) mice, using a previously described (Section 2.2.1). Bacteria used as a 'dead' control were exposed to  $1 \times 10^6$  rad gamma irradiation in a Shepherd Model 484 irradiator (J. L. Shepherd and Associates, Fernando, CA) using a previously described protocol (Adams, L.B. *et al*, 2000). Both live and irradiated bacteria were held overnight at 4<sup>0</sup> C and then used to inoculate Schwann cell cultures. Viability of the freshly harvested bacteria was verified by BACTEC analysis as previously described (Section 2.2.1)

### **3.2.2. Schwann Cell Cultures**

Schwann cells were purified from the sciatic nerves of neonatal Holtzman outbred rats (HsdHot:Holtzman<sup>J</sup> SD, Harlan, Indianapolis, IN) using a modified Brockes method (Brockes, J.P. *et al*, 1979; Kleitman, N. *et al*, 1996) as described in Chapter 2. To produce large numbers of Schwann cells for use in co-culture experiments, Schwann cells were mitotically expanded using characterized Schwann cell mitogens (Section 2.2.2.)

### **3.2.3. Purity and Viability of Schwann Cell Cultures**

The purity of Schwann cell cultures was determined by immunostaining analysis for the presence of the Schwann cell marker, S100 cell surface protein (Pelc, S. *et al*, 1986), using S100 antibody (Sigma Chemical Co., St. Louis, MO) as described in (Section 2.2. 3.). Cultures were considered pure when fibroblast contamination was  $\leq 5\%$  the total cell population. Trypan blue dye exclusion analysis (Life Technologies, Rockville, MD) was used to determine the viability of these cell preparations.

### **3.2.4. Infection of Schwann Cells**

Four days prior to inoculation, Schwann cells were plated in D-10 containing forskolin and bovine pituitary extract. Half of the cultures were then placed at 33<sup>0</sup> C 24 h prior to infection. Schwann cells were infected with freshly harvested *M. leprae* at a multiplicity of infection of 100 *M. leprae* per Schwann cell (MOI = 100) and exposed to these bacteria for 48 h at either 33<sup>0</sup> C or 37<sup>0</sup> C in 5% CO<sub>2</sub> as previously described (**Section 2.2.6.**). The bacterial preparation was passed through a 27 ½gauge needle prior to inoculation to reduce bacterial clumping. Fresh medium was replenished twice weekly.

### **3.2.5. *M. leprae* Viability in Schwann Cells**

The metabolic activity of *M. leprae* in Schwann cells was determined one week post-infection using a modification of the Buddemeyer radiorespirometry method (Franzblau, S.G. *et al*, 1992) as described in **Section 2.2.7.** Metabolic activity was reported as counts per minute (CPM).

### **3.2.6. Purification of RNA and Reverse Transcriptase-PCR**

Total RNA was purified from individual wells of tissue culture plates containing Schwann cell monolayers using the following protocol. The medium was removed and cells were lysed by the addition of 300 µl guanidinium HCL (RNAgents Total RNA Isolation System, Promega, Piscataway, NJ). RNA was extracted from these cell lysates using the manufacturer's recommendations and stored at -70<sup>0</sup> C until used. Primers and parameters for semi-quantitative analysis were performed as described in **Section 2.2.9, Table 3.1 and 3.2.**

### **3.2.7. Statistical Analysis of Data**

All experiments were performed in triplicate and data were expressed as the mean and standard deviation of at least three replicates per experiment. All data were analyzed using SAS

analysis of variance in a factorial arrangement of treatment effects (temperature, live *M. leprae*, dead *M. leprae* and non-infected controls). Post hoc analyses were conducted with pairwise-t-test comparisons of least square means and all probabilities were considered significant at ( $p \leq 0.05$ ).

BACTEC GI levels of  $> 100$  were obtained for all bacterial harvests used in these experiments, indicating that these preparations contained highly viable *M. leprae* (data not shown). Buddemeyer radiorespirometry data indicated that the *M. leprae* kept at  $4^{\circ}\text{C}$  overnight prior to inoculation were as metabolically active when held in Schwann cells at  $33^{\circ}\text{C}$  as *M. leprae* which were immediately used after harvesting (**Fig 3.1.**). This analysis also revealed that irradiated *M. leprae* kept at  $4^{\circ}\text{C}$  overnight prior to infection of Schwann cells at  $33^{\circ}\text{C}$  did not contain any metabolic activity within one week of infection. In addition, the phagocytic index (PI) was similar to that observed for non-induced Schwann cells (data not shown).

**Table 3.1.** Primer sequences for use in polymerase chain reaction (PCR) semiquantitative analysis of cDNA collected from samples.

Schwann Cell Marker	Primer Sequence
G3PDH	Clontech Catalog # 5405-1
GFAP	5'ACTGCAGGCCTTGACCTG3' 5'GATCACCTGCCCATCAGG3'
ICAM	5'CAGTAGTGCTGCTCC3' 5'GAGCTTCAGAGGCAG3'
L1	5'TCAGACTACATCTGCAATGC3' 5'CATTACTCAGGATCAAAGACC3'
N-cadherin	5'ATCTATGAAGTTCCCATCG3' 5'TCCAAAAGCCAAGTCC3'
NCAM	5'AGAACATCCCTCCCAGCC3' 3'GCGTTGTAGATGGTCAGGGT3'
TGF $\beta$ 1	Clontech Catalog # 5501-1



**Table 3.2.** Polymerase Chain Reaction (PCR) parameters for primer sets (Table 3.1) used for semiquantitative reverse transcription-PCR analysis. All sets were denatured at 94<sup>0</sup> C for 7 minutes; then cycled at 94<sup>0</sup> C, annealing temperature and 72<sup>0</sup> C for the time parameters specified (respectively); and finally placed at 72<sup>0</sup> C for 10 minutes for extension.

Primer	Primer Concentration	Annealing Temperature	Number of Cycles	Cycling Parameters (minutes)	Anticipated Fragment Size
G3PDH	20μM	64 <sup>0</sup> C	25	1, 1, 1	452bp
GFAP	10μM	62 <sup>0</sup> C	35	1, 1, 1	340bp
ICAM	10μM	67 <sup>0</sup> C	30	0.5, 0.5, 0.5	240bp
L1	5μM	55 <sup>0</sup> C	30	1, 1, 1	629bp
N-cadherin	5μM	55 <sup>0</sup> C	30	1, 1, 1	759bp
NCAM	10μM	60 <sup>0</sup> C	35	1, 1, 1	280bp
TGFβ1	20μM	60 <sup>0</sup> C	28	0.5, 0.5, 0.5	525bp

### 3.3. Results

Schwann cell monolayers produced the typical ‘swirled’ appearance under all conditions evaluated until 12 d post-infection (**Fig. 3.2.**). At this time, Schwann cells infected with metabolically active *M. leprae* at 33<sup>0</sup> C lost the ‘swirled’ monolayer appearance. Cytotoxicity analysis revealed that alteration of the monolayer morphology was not due to cell death (data not shown).

Analysis of gene transcription in Schwann cells infected with live or dead *M. leprae* at 33<sup>0</sup> C revealed that the expression of several Schwann cell genes was altered 12 d post infection in some cultures compared to that of noninfected control cultures. GFAP mRNA levels were significantly ( $p > 0.05$ ) down-regulated in cultures infected with both live and dead bacilli (**Table 3.1.**). NCAM mRNA levels were significantly up-regulated ( $p \leq 0.05$ ) in cultures infected with live bacilli. TGF-β1 mRNA levels did not appear to be altered when cultures were infected with live *M. leprae* but were significantly lower when the cultures were exposed to dead

*M. leprae*. N-cadherin transcripts were also significantly lowered within live *M. leprae*-infected Schwann cells at 33<sup>0</sup> C. L1 and ICAM transcripts did not appear to be significantly altered.

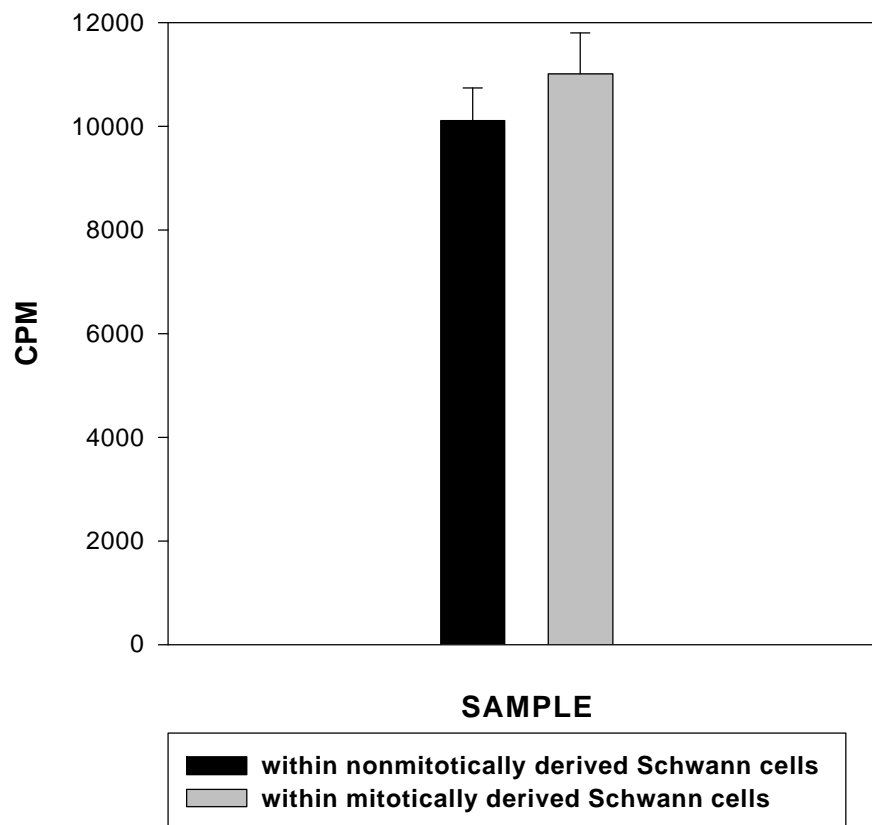
### **3. 4. Discussion**

Mitogen-expanded primary Schwann cells were evaluated for their suitability in the improved model to study *M. leprae* Schwann cell interactions. One of the main benefits of using these cells is that they replicate every 2 – 5 days instead of every 7-8 days. Therefore, more cells are available in a shorter period of time for experiments. In addition, because they replicate so rapidly they out grow the low levels of fibroblasts in the population and thereby, typically produce cultures with higher purity. Mitogen-expanded primary Schwann cells have been shown to retain many of the important primary Schwann cell characteristics and are capable of interacting with axons in a similar manner to non-induced cells *in vitro* (Seth Porter, M. *et al*, 1986; Rahmatullah, M. *et al*, 1998; Li, H. *et al*, 1998). Therefore, when these cells were evaluated for use in the improved model for *M. leprae* infection studies.

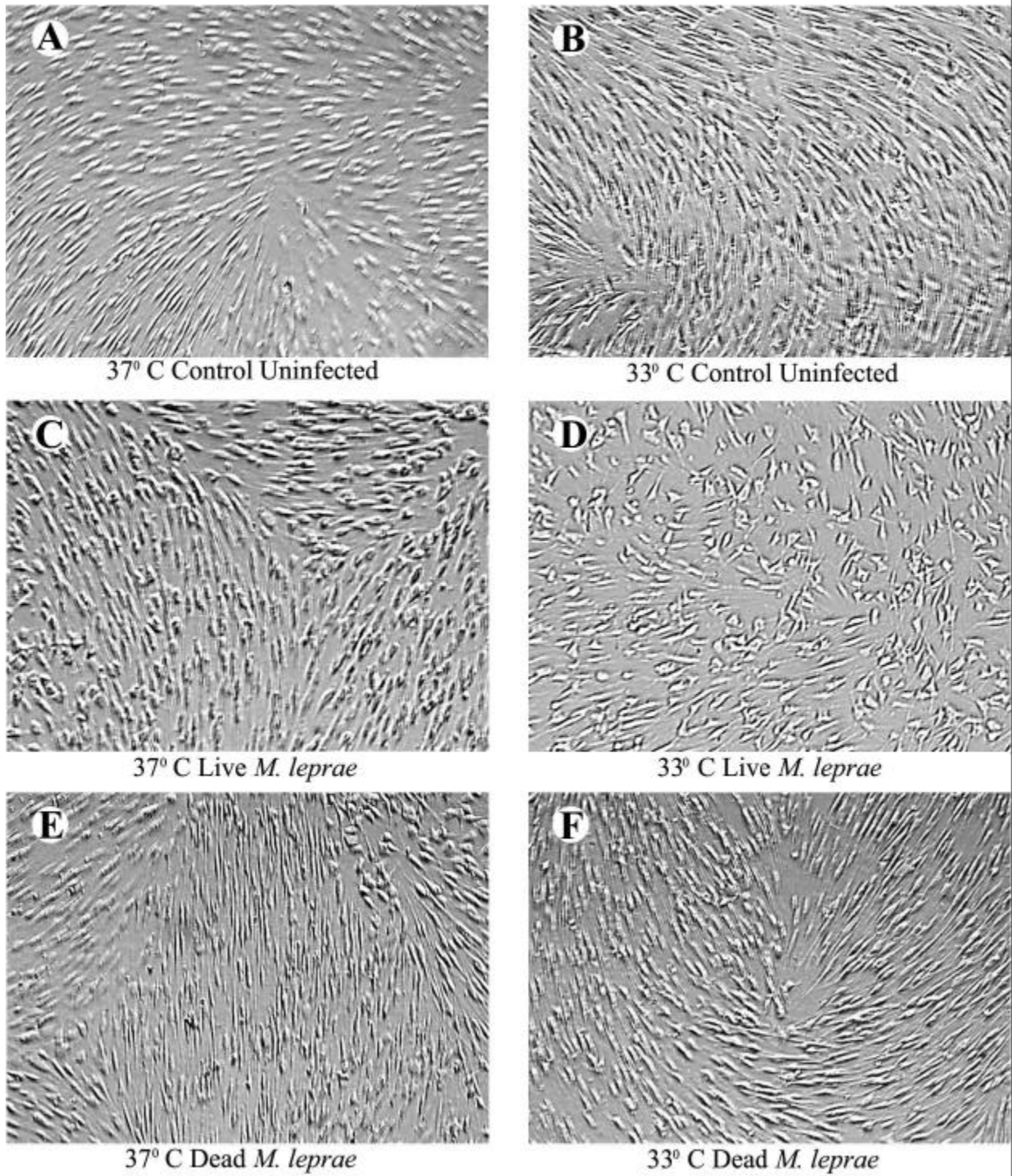
Primary Schwann cells cultured in the presence of 100 MOI live *M. leprae* at 33<sup>0</sup> C have previously been shown to phagocytize *M. leprae* within 48 h resulting in a phagocytic index (PI) of  $20 \pm 5$  and > 99% infection of the culture (**Section 2.3**). A similar PI and % infection level was observed in this study with the mitogen-expanded cultures, indicating that mitogen treatment did not alter the ability of the Schwann cells to phagocytize *M. leprae*.

Primary Schwann cells cultured in the presence of live *M. leprae* at 33<sup>0</sup> C have previously been shown to develop morphological alterations 4-6 d post infection (**Section 2.3**). In the present report, we demonstrate that mitotically-expanded Schwann cells also respond to live *M. leprae* infection with morphological alterations at 33<sup>0</sup> C. Cells clumped in non-induced cultures resulting in the loss of the typical ‘swirled’ monolayer morphology, whereas, no apparent

clumping was observed in the mitogen-expanded cultures. However, loss of the typical ‘swirled’ monolayer was consistently observed in cultures infected with metabolically active *M. leprae* at 33<sup>0</sup> C. As in the non-induced cultures, the loss of ‘swirled’ monolayer morphology indicated an altered ability of the Schwann cells to interact with one another and/or the substrate.



**Figure 3.1.** *M. leprae* metabolic activity after overnight 4<sup>0</sup> C incubation before exposure to mitotically-expanded Schwann cells compared to metabolic activity of freshly harvested *M. leprae* exposed to primary Schwann cells. *M. leprae* were maintained within mitotically-expanded and non-mitotically expanded Schwann cells at 33<sup>0</sup> C for one week and then analyzed for metabolic activity by Buddemeyer radiorespirometry (counts per minute, CPM).



**Figure 3.2.** Scanning electron micrographs of mitotically-expanded primary Schwann cell monolayers 12 d post-infection with *M. leprae*. *M. leprae* was harvested from nude mouse footpads, held overnight at 4° C and then exposed to mitotically-derived Schwann cells at either 33° C or 37° C. Irradiated (dead) *M. leprae* and uninfected Schwann cell monolayers were used as controls. Micrographs shown at approximately 40x final magnification.

**Table 3.3.** Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of Schwann cell gene transcripts 12 d post-infection with *M. leprae* from control (uninfected), *M. leprae*-infected (live) and cultures exposed to irradiated *M. leprae* (dead) maintained at 33<sup>0</sup> C. PCR products were quantitated by scanning agarose gels and the use of densitometry analysis. Sample IODs (Integrated Optical Density) were normalized by dividing sample IODs by that of G3PDH (constitutive housekeeping gene) for the same sample to give normalized IOD (nIOD).

Marker	Control nIOD	Live nIOD	Dead nIOD
GFAP	1.52 ± 0.15	1.06 ± 0.15*	1.17 ± 0.15*
TGFβ1	1.52 ± 0.18	1.25 ± 0.18	0.86 ± 0.18*
NCAM	0.67 ± 0.22	1.39 ± 0.22*	0.85 ± .022
ICAM	0.33 ± 0.08	0.57 ± 0.08	0.32 ± 0.08
N-Cadherin	2.45 ± 0.50	0.91 ± 0.50*	1.20 ± .050
L1	0.81 ± 0.26	0.49 ± 0.26	0.65 ± 0.26

“\*” denotes that values were significantly different ( $p \leq 0.05$ ) when compared to control

Mitogens in the medium during infection may have influenced these Schwann cells to respond differently to infection. It is well known, though not very well understood, that the common Schwann cell mitogens: forskolin, bovine pituitary extract and heregulin substitute for some of the axonal signaling that Schwann cells commonly receive when in direct contact with an axon. For instance, protein zero (P0) transcripts, which are typically only produced by myelinating Schwann cells, are produced by primary Schwann cells under the influence of mitogens (LeBlanc, A.C. *et al*, 1992). It is, therefore, plausible that the mitogens, imitating axonal signals for the Schwann cells, may have influenced the degree of morphological response exhibited by the Schwann cells.

The metabolic activity of *M. leprae* in these experiments was also analyzed and found to be comparable to that of bacteria in non-induced Schwann cells held at 33<sup>0</sup> C (**Section 2.3.**), even though the bacterial preparation was held overnight at 4<sup>0</sup> C prior to infection. These data indicated that 4<sup>0</sup> C incubation for 24 h prior to infection does not appear to alter the metabolic activity of the *M. leprae* inoculum. In addition, the response of Schwann cells to stimulation with mitogens does not appear to be detrimental to the intracellular viability of *M. leprae*.

Gene expression analysis and morphological analysis of mitogen-expanded Schwann cells indicated that these cells responded to 33<sup>0</sup> C and to that of non-induced cells (**Section 2.3.**). Therefore, these data suggested that mitogen-stimulated Schwann cells were suitable for the study of *M. leprae*/Schwann cell interactions.

Infection of cultures at 33<sup>0</sup> C induced altered expression of some Schwann cell genes encoding typical Schwann cell markers routinely used to identify the effects of environmental factors on short term-cultured Schwann cells. Elevated levels of NCAM transcripts and reduced levels of GFAP transcripts were observed in these cultures. These molecules are both important mediators of Schwann cell/Schwann cell and Schwann cell/axon interactions. In addition, NCAM is an important adhesion molecule in the development and maintenance of myelin sheaths. GFAP has been shown to be important for glial cell process formation in the central nervous system (Chen, W.J. and Liem, K.H., 1994) and most likely has the same function in the Schwann cells of the peripheral nerves.

TGFβ1 gene expression was not significantly altered by intracellular, metabolically active *M. leprae*; however, dead bacilli were capable of down-regulating TGFβ1 expression. Irreversible nerve damage has also been shown to continue to develop during and even after antimicrobial therapy has killed the bacteria within leprosy patients (Gillis, T.P and Hasting, R.C., 1992; Rambukkana, A., 2000). It is estimated that therapeutic intervention has prevented only one-third of infected individuals from suffering further nerve damage. The decrease of TGFβ1 transcripts within the Schwann cells containing dead *M. leprae* may indicate that the intracellular presence of the dead bacteria may suppress the Schwann cell's ability to mediate response to trauma or damage by TGFβ1. Therefore, dead bacilli in lesions may also contribute

to neuropathology of leprosy. These data also demonstrate the need for the use of both viable and dead *M. leprae* within the standardized infection model.

N-cadherin gene transcripts were also shown to significantly decrease within metabolically active *M. leprae*-infected Schwann cells by 12 d post-infection. Since N-cadherin has been shown to be localized on the plasma membranes at points of contact between both axons and Schwann cells but not basal lamina (Shibuya, Y. *et al*, 1995), altered expression this molecule may lead to altered Schwann cell/Schwann cell and Schwann cell/neuron interactions.

If altered levels of mRNAs encoding these markers are indicative of altered protein concentrations of these molecules in infected Schwann cells, then altered concentrations of these markers may be at least somewhat responsible for the alteration of Schwann cell monolayer morphology. Further analyses of surface molecule expression of N-cadherin and other important adhesion molecules relevant to Schwann cell/Schwann cell interaction by immunofluorescent microscopy could potentially be used to determine if the observed gene alterations actually result in the expression of the altered morphology observed.

In addition, alteration of important adhesion molecules and GFAP transcripts may have an effect on the ability of Schwann cells to interact appropriately with axons and/or other Schwann cells. This could have effects on the ability of these Schwann cells to produce and maintain myelin as well as to properly participate in regenerative processes to repair peripheral nerve damage.

L1, ICAM and NCAM are highly expressed during development by both the neuron and the Schwann cell, down-regulated postnatally and re-expressed during axonal regeneration after injury *in vivo*. L1 gene expression levels in our experiment were not altered while NCAM gene expression levels were significantly increased 12 d post-infection with metabolically active *M.*

*leprae*. These two neural cell adhesion molecules interact homophilically with similar molecules on associated Schwann cells and neurons (Martini, R. *et al*, 1994) and both have critical roles in the Schwann cell and axon relationships. Zhang, *et al* (2000) have shown that after injury *in vitro* L1 gene expression within neurons is not increased. Martini and Schachner (1988) demonstrated that both molecules are up-regulated on the surface of Schwann cells/Schwann cell and Schwann cell/axon interfaces during regeneration.

In order to determine the effects of *M. leprae* on Schwann cell functional capabilities, further studies are necessary. Even though significant alterations in gene transcript levels may indicate potential alteration of production of these molecules, the functional aspects of *M. leprae*-infected Schwann cell's ability to properly relate with axons and produce myelin sheaths may not be altered. In order to determine this, comprehensive electron microscopic analyses must be conducted to determine the relationship between infected Schwann cells and axons, as well as the effects of infection on myelin production, architecture and maintenance.

In this report, we demonstrate the usefulness of mitogen-expanded Schwann cells in the improved infection model and show that they respond similarly to infection as non-induced cells. The data presented suggest that these cells are suitable for *in vitro* infection studies. When these cells were infected using the parameters of the 33<sup>0</sup> C infection model described in **Section 2.2.2** and used to determine the effects of infection on specific Schwann cell adhesion genes, data indicated that significant gene expression alteration does occur as a result of metabolically active *M. leprae* infection. In addition, this study demonstrates that dead organisms may also contribute to adverse effects upon the Schwann cell *in vivo*.



## **CHAPTER 4.**

### **EFFECTS OF *MYCOBACTERIUM LEPRAE* ON SCHWANN CELL FUNCTION AND SCHWANN CELL/NEURON INTERACTIONS USING MICROSCOPIC ANALYSES**

#### **4.1. Introduction**

*Mycobacterium leprae*, the causative agent of leprosy, is the only bacterium known to specifically target the Schwann cell of the peripheral nervous system (Lumsden, C., 1959; Stoner, G.L., 1979; Mukherjee, R. and Antia, N.H., 1986; Job, C.K., 1989). Clinical evidence of disease is usually characterized by nerve paralysis, anaesthesia, tingling, numbness and burning sensations, thickening of nerves, and occasional muscle weakness (Chimelli, L. *et al*, 1997).

Light and electron microscopic studies of leprosy patient nerve biopsies have shown that a large number of infected Schwann cells undergo degeneration and destruction. This apparently results in demyelination, axonal damage and irreversible destruction of nerve architecture (Job, C.K., 1971). In one study of patient nerve biopsies, early stages of disease were associated with normal histological appearance of the nerves with slight edema; however, as the bacillary load within Schwann cells increased, Schwann cells appeared to degenerate (Job, C.K., 1971). Other studies found few common neuropathological features among samples of various stages of disease (Jacobs, J.M., 1987a). Edema and 'loosened' myelin were frequently observed, and all of these studies described focal regions of total demyelination often occurring in relation to cellular infiltration during latter stages of infection (Jacobs, J.M., 1987b); however, the early mechanisms of leprosy neuropathology are not well understood (Spierings, E. *et al*, 2001). In addition the direct effect of *M. leprae* infection on Schwann cells is not understood.

It is therefore conceivable that *M. leprae* infection of the Schwann cell may directly result in the alteration of Schwann cell function and/or affect the critical relationship of the Schwann cell with the axon. This may alter the Schwann cell's ability to mount or participate in

a regenerative response or the capability to produce and maintain myelin. Mukherjee *et al* (1981) reported Schwann cells infected with *M. leprae* failed to interact with axons and did not proceed to produce myelin *in vitro* as shown by both light and electron microscopy. However, these experiments were performed at 37<sup>0</sup> C using *M. leprae* harvested from the nodules of untreated lepromatous leprosy patients. Because no viability analysis was performed on *M. leprae* used as inoculum for these experiments, these conclusions may not reflect the effects of viable *M. leprae* infection of the Schwann cell (**Chapter 2**). Therefore, to date, no comprehensive, *in vitro* electron microscopic studies have been conducted to determine the effects of confirmed viable *M. leprae* infection on Schwann cells or on Schwann cell/ neuron interactions and myelination.

It is known that *M. leprae in vivo* is found in the cooler regions of its host and it is in these regions that *M. leprae* replicate and exhibit the most destruction. We have recently shown that temperature has a critical impact on the viability of *M. leprae* in Schwann cells maintained *in vitro* and *M. leprae* remain metabolically active longer at 33<sup>0</sup> C than at 37<sup>0</sup> C. This information has been used to develop a new infection model that utilizes freshly harvested, highly viable *M. leprae* and 33<sup>0</sup> C incubation temperature during infection (**see Chapter 2**).

Using this model to study *M. leprae*/Schwann cell interactions, it was determined that *M. leprae* held within the Schwann cells at 33<sup>0</sup> C for up to 21 d retained 56% of its initial metabolic activity. In addition, infected Schwann cells exhibited morphological alterations at the light microscope level as a result of infection (**Chapter 2**) as well as alteration in gene expression for several key adhesion molecules such as neural-cadherin (N-cadherin), neural cell adhesion molecule (NCAM), and glial fibrillary acidic protein (GFAP) (**Chapter 3**). These molecules are critical to the Schwann cell/neuron relationship and regenerative process.

Therefore, in the present study, electron microscopy and the 33<sup>0</sup> C co-culture infection model were used to investigate the effects of *M. leprae*-infection on Schwann cell morphology and the ability of these cells to appropriately interact with neurons in co-culture. In addition, the effects of infection on myelin architecture were studied.

## **4.2. Materials and Methods**

### **4.2.1. Bacteria**

*M. leprae* Thai-53 were purified from the hind foot pads of nude BALBc (*nu/nu*) mice, as previously described in **Section 2.2.1**. Viability analysis was performed using the BACTEC 460 system (**Section 2.2.1**). Bacteria from the same harvests were exposed to 1 x 10<sup>6</sup> rad gamma irradiation in a Shepherd Model 484 irradiator (J.L. Shepherd and Associates, Fernando, CA) using a previously described protocol (Adams, L.B. *et al*, 2000).

### **4.2.2. Schwann Cell Cultures**

Schwann cells were purified from the sciatic nerves of neonatal Holtzman outbred rats (HsdHot:Holtzman<sup>J</sup> SD, Harlan, Indianapolis, IN) using a modified Brockes' method (Brockes, J.P. *et al*, 1979; Kleitman, N. *et al*, 1996) (**Section 2.2.2**.) To produce large numbers of Schwann cells for use in co-culture experiments, Schwann cells were mitotically expanded using characterized Schwann cell mitogens, as described in **Section 3.2**.

### **4.2.3. Purity and Viability of Schwann Cell Cultures**

The purity of Schwann cell cultures was determined by immunostaining analysis for the presence of the Schwann cell marker, S100 cell surface protein (Pelc, S. *et al*, 1986), using anti-S100 antibody (Sigma Chemical Co., St. Louis, MO), as described in **Section 2.2.3**. Cultures were considered pure when fibroblast contamination was  $\leq 5\%$  the total cell population. Trypan

blue dye exclusion analysis (Life Technologies, Rockville, MD) was used to determine the viability of these cell preparations.

#### **4.2.4. Neuron Cultures**

Neurons were dissociated and purified from dorsal root ganglia (DRG) harvested from day 15 embryonic rats and plated on to Thermanox coverslips (Nalge Nunc International, Naperville, IL) as described in **Section 2.2.4**. All cells were incubated at 37<sup>0</sup> C until axonal sprouts extended from neurons and thickened and cultures were relatively free of other contaminating cell types (3-4 weeks).

#### **4.2.5. Schwann Cell/Neuron Co-Cultures**

Monolayers of purified Schwann cells were dissociated with trypsin-EDTA, seeded onto purified neurons and stimulated to myelinate in the presence of ascorbate (**Section 2.2.5**). All cultures were incubated at 33<sup>0</sup> C or 37<sup>0</sup> C for 24 h prior to and during infection.

#### **4.2.6. Infection of Schwann Cells and Co-cultures**

Schwann cells were infected with *M. leprae* at a multiplicity of infection (MOI) = 100:1 and exposed to these bacteria for 48 h at 33<sup>0</sup> C in 5% CO<sub>2</sub> after which >99% cells were shown to be infected (0 d post-infection; Section 2.3.) Medium was removed, monolayers were washed with D-10 and the cells were incubated up to 21 days post infection. Media was replenished every three days.

Schwann cells were dissociated with trypsin-EDTA at either 4 or 12 d post infection with *M. leprae*. Approximately 5x 10<sup>4</sup> cells in 1 ml neuralbasal (NB1)/D10 (1:1) were seeded onto purified neurons cultured on plastic coverslips in 24-well plates. Cells were allowed to attach, align and proliferate along axons. Co-cultures were induced to myelinate by addition of ascorbate as described in **Section 2.2.5**.

To determine the effects of *M. leprae* on intact, myelinated Schwann cell/axon units, one-half of myelinated co-cultures (**Section 4.2.6.**) were shifted to 33<sup>0</sup> C for 24 h prior to infection. Co-cultures were then infected with *M. leprae* at an MOI = 100. Cultures were incubated for 48 h, washed and maintained up to 30 d post infection. Media was changed three times per week.

#### **4.2.7. *M. leprae* Viability in Schwann Cells**

The viability of *M. leprae* in Schwann cells was determined indirectly using a modification of the Buddemeyer radiorespirometry method (Franzblau, S.G. *et al*, 1992) as described in **Section 2.2.7.**

#### **4.2.8. Microscopy**

Coverslips containing co-cultures were removed from tissue culture plates, fixed and either processed for scanning electron microscopy (SEM) or transmission electron microscopy (TEM) as described in **Section 2.2.8.** In addition, thick sections were obtained from blocks and were acid fast stained using a previously described procedure (Luderschmidt, C. 1987).

To determine the effects of *M. leprae* infection on Schwann cell and Schwann cell/neuron co-cultures in these experiments, several palladium-coated studs containing these co-cultures and grids containing thin sections that represented cells in each experimental condition were observed using scanning electron microscopy and transmission electron microscopy, respectively (**Section 2.2.10.**). At least 25 cells were observed for each of the experimental conditions.

### **4. 3. Results**

Microscopic analyses were used to determine the effects of *M. leprae* infection on the ability of the Schwann cell to 1) align with axons; 2) associate with axons; 3) proliferate along axons; 4) produce myelin and 5) maintain myelin sheath architecture in SC/axon units. In

addition, the effects of infection on Schwann cell morphology and intracellular structures were determined.

Results demonstrated that Schwann cells infected with either irradiated or viable *M. leprae* and seeded onto purified neurons were capable of attaching and aligning with axons or cultured neurons within 24 h in a manner comparable to uninfected controls at both 33<sup>0</sup> C and 37<sup>0</sup> C (**Figures 4.1. and 4.2.**) In addition, infected Schwann cells proliferated along the axons in a seemingly normal manner (**Figure 4.3.**). Thick section acid-fast stain microscopy verified the intracellular presence of *M. leprae* within these co-cultures and Buddemeyer radiorespirometry analysis indicated that the *M. leprae* in cultures infected with viable *M. leprae* were still highly viable (data not shown).

When infected co-cultures were stimulated with ascorbic acid and allowed to myelinate, it was found that all co-cultures contained myelinating and nonmyelin-producing Schwann cells (**Figs. 4.4., 4.5 and 4.6.**). Schwann cells containing *M. leprae* were capable of ensheathing axons of various sizes.

The Schwann cell/axon membrane interfaces of infected cells appeared to be contiguous and comparable to those in controls demonstrating that infection of Schwann cells prior to seeding onto axons did not appear to alter their ability to appropriately associate with axons (**Figs. 4.4., 4.5. and 4.6.**). In addition infected Schwann cell surface and nuclear membranes were intact and similar to those observed in controls.

Surface membranes of axons appeared to be intact and comparable to controls, and no *M. leprae* were observed within axons (**Fig. 4.4. - 4.8.**). No differences were observed in microtubule formation or mitochondrial structures.

Infected Schwann cells appeared to produce basal lamina and myelin sheaths similar to those seen in the controls. Schwann cells exhibited varying stages of myelin sheath formation as well as the thickness of myelin sheaths appeared to be related to the diameter of the ensheathed axon. No differences, such as, “loosening” of the myelin lamellae were observed in any of the infected cells (**Fig. 4.7.**).

Established myelinated Schwann cell/neuron co-cultures were also infected with freshly harvested, viable *M. leprae* and held at 33<sup>0</sup> C for up to one month to investigate the functional response of Schwann cells associated with an axon to infection. Phase contrast microscopy of cultures maintained, one month post infection, indicated that there were no overt morphologic changes in these cultures when compared to control cultures (**Fig. 4.9.**)

The results of this study showed that *M. leprae* were found in both myelinated and nonmyelin-producing Schwann cells in these cultures. Either single bacillus or clumps of bacilli were located within vacuoles in the cytoplasm of all infected cultures analyzed (**Fig. 4.10. and 4.12.**).

Lipid-like vacuoles were observed within Schwann cells in all culture conditions (**Fig. 4.11.**). There were no apparent differences in the appearance of these vacuoles in any of the Schwann cell groups evaluated.

The Schwann cells appeared to be morphologically similar and no changes in myelin architecture were apparent when compared to uninfected controls (**Fig. 4.10.**). In addition, radiorespirometry indicated that live infected co-cultures held at 33<sup>0</sup> C retained 55% of *M. leprae* metabolic activity as compared to 0% at 37<sup>0</sup> C after one month (data not shown).

The Schwann cell/axon membrane interfaces of both infected myelinating and non-myelin producing cells in these cultures were contiguous and comparable to uninfected controls.

Schwann cell surface membranes, basal lamina formations and organelles and nuclear membranes were comparable in infected and uninfected co-cultures. Lipid-like bodies, similar to those seen in the infected Schwann cell seeding assay, were also observed within Schwann cells in all conditions within this experiment.

Myelin thickness and integrity were similar in both infected and uninfected co-cultures. No aberrant formation, “loosening”, of myelin lamellae or myelin destruction was observed within infected co-cultures. (**Fig. 4.10.**).

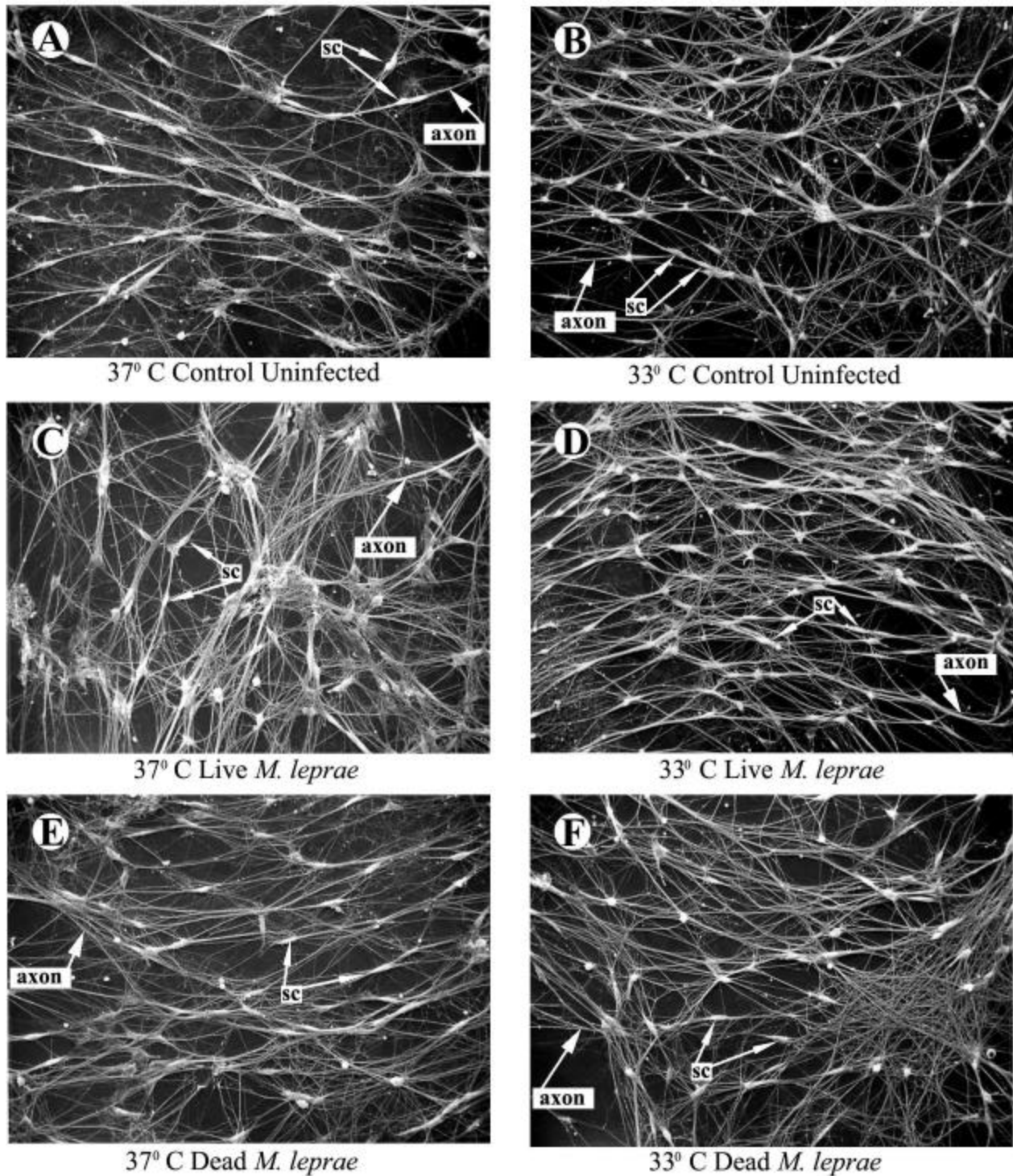
No *M. leprae* was observed within axons or neuronal bodies; even though axons that were not ensheathed by Schwann cells had been exposed to freshly harvested viable *M. leprae* for 48 h during the Schwann cell infection process. Axonal surface membranes, mitochondria, and microtubules were comparable in all co-cultures.

#### **4. 4. Discussion**

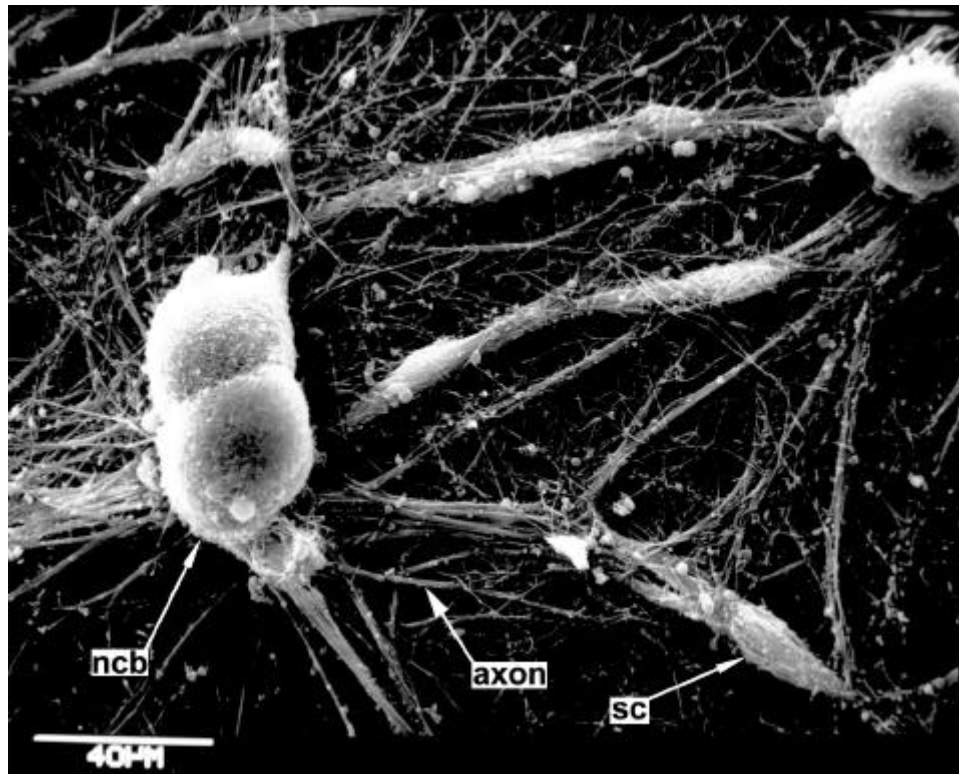
It has been previously demonstrated that *M. leprae* bind the laminin  $\alpha$ -2 chain of the basal lamina of the Schwann cell-axon unit to specifically target the Schwann cell of the peripheral nerve system (Ng, V. *et al*, 2000). Once intracellular, the mechanisms by which neuropathology develops are not well understood (Shetty, V.P. *et al*, 1994; Spierings, E. *et al*, 2001; Job, C.K., 1971). Evidence suggests that neuropathology could be attributed to the direct effect of intracellular *M. leprae* upon the Schwann cell or a combined effect with the immune response to infection (Jacobs, J.M. *et al*, 1987; Job, C.K., 1971).

In this study, Schwann cells within monolayers which had exhibited morphological and gene expression alterations as a result of infection with *M. leprae* (**Sections 2.3 and 3.3**) did not exhibit any apparent functional alterations when seeded onto neurons in culture at 33<sup>0</sup> C. It is possible that the altered gene expression did not necessarily translate into altered protein





**Figure 4.1** Scanning electron micrographs of *M. leprae*-infected primary Schwann cells 24 h post-seeding onto purified neurons at either 33° C or 37° C. Metabolically active (live) or irradiated (dead) *M. leprae*-infected Schwann cells and uninfected (control) Schwann cells (SC) that had been maintained for 12 d at either temperature were trypsinized, seeded onto neurons *in vitro* and then maintained at their respective temperatures. Micrographs shown at approximately 150x final magnification.

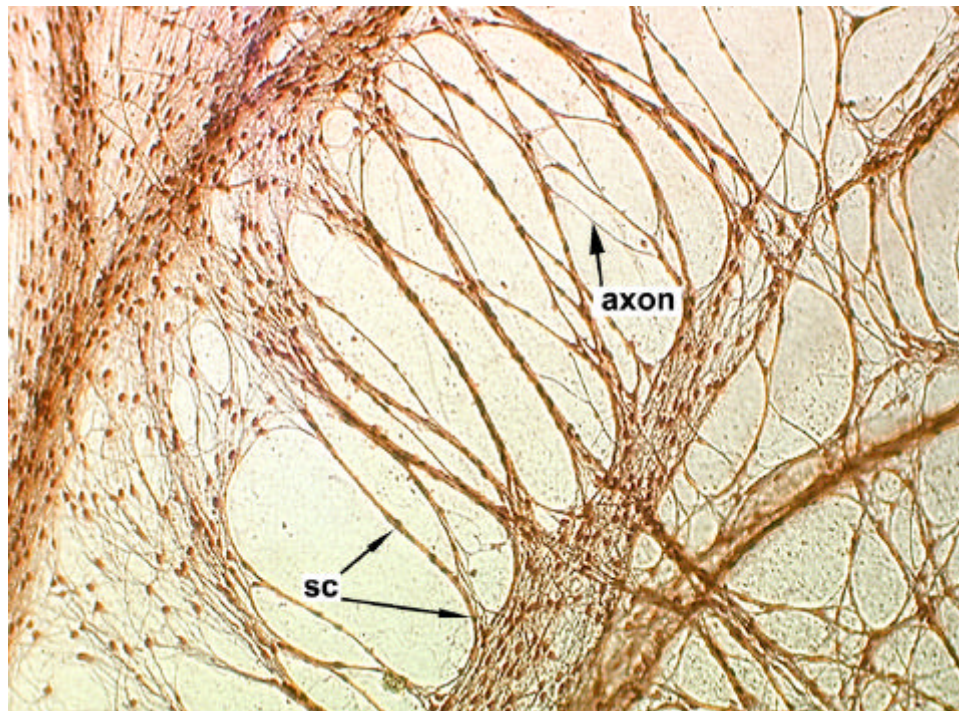


**Figure 4.2.** Scanning electron micrograph of Schwann cells (SC) infected with live *M. leprae*, maintained at 33<sup>0</sup> C for 12 d, seeded onto purified neurons and held for 24 h at 33<sup>0</sup> C. NCB = neural cell bodies.

production or that the alteration of protein concentration was insufficient to interfere with normal Schwann cell/neuron interactions and subsequent myelin production and maintenance.

It is possible that potential alterations of cell surface molecules may have been affected when the Schwann cell monolayers were dissociated by trypsin prior to seeding onto neuron. Previous studies have shown that Schwann cell surface protein molecules can be altered by treatment with trypsin (Sasagasako, N. *et al*, 1999; Itty, B.D. *et al*, 1986; Sobue, G. and Pleasure, D., 1985); however in this study, cells were maintained for one month post-seeding at which point no gross differences were observed between cultures regardless of infection status or temperature.

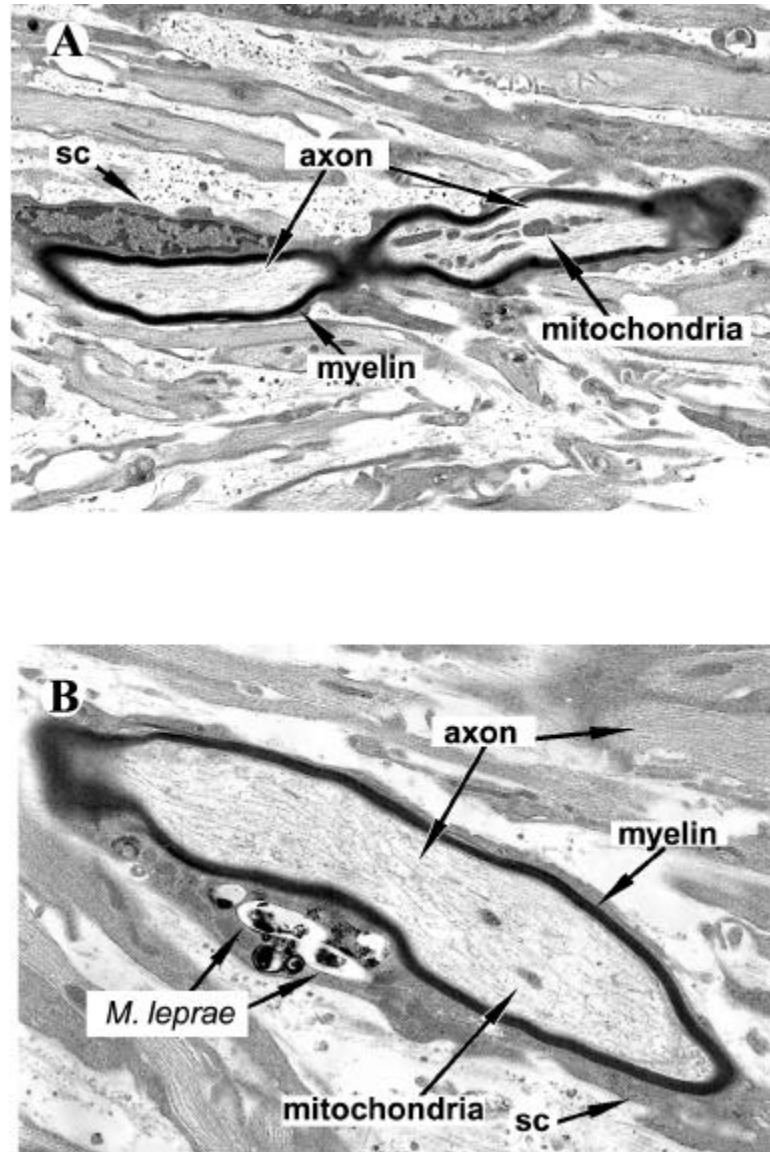
No apparent effects of infection were observed within infected Schwann cells in co-cultures even though some of these cells contained large numbers of bacteria (>10 bacilli). In addition, no differences were observed in Schwann cells whether they were infected prior to or after association with an axon.



**Figure 4.3.** Light microscopy of anti-S100 immunostain of primary Schwann cell/neuron co-culture approximately 2 weeks post-seeding after the Schwann cells (SC) had proliferated along the axons. Micrograph taken at approximately 40x magnification.

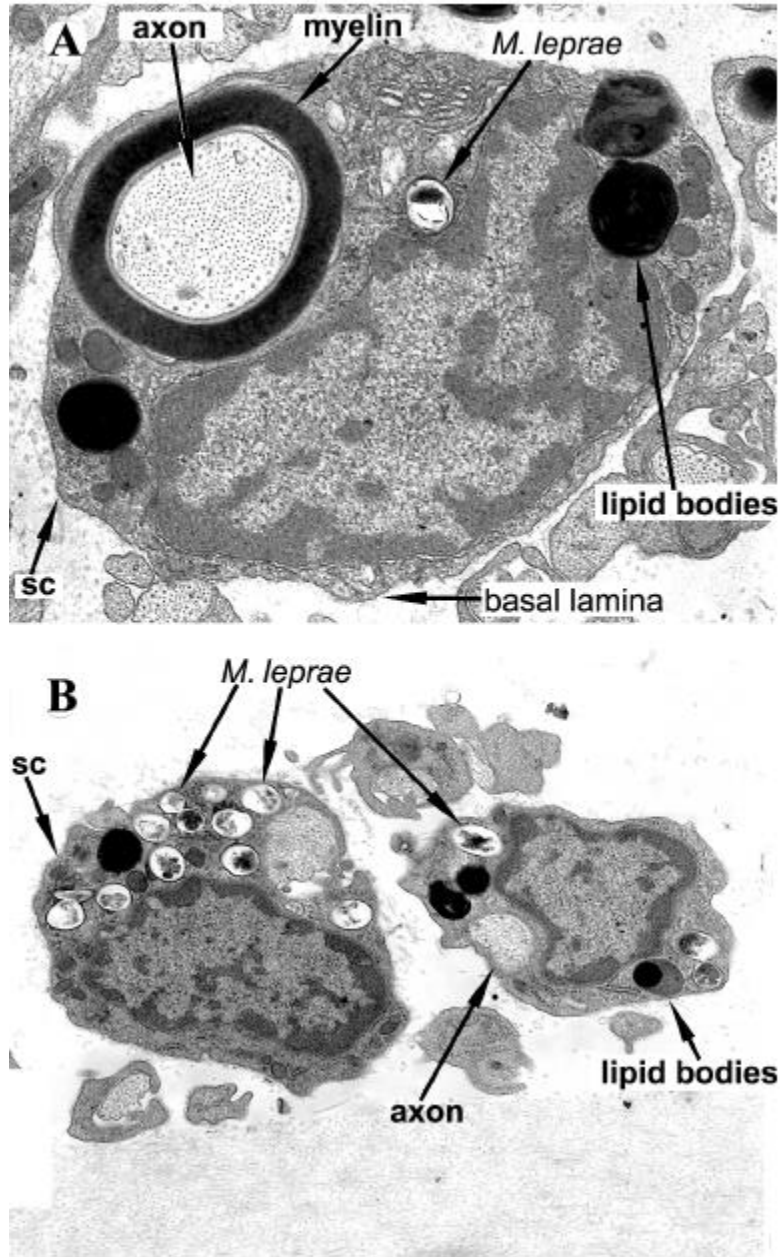
Although specific assays were not performed to quantitate basal lamina formation within these cultures, electron micrographs of Schwann cells in co-culture with neurons did exhibit basal lamina (Figure 4.). It has also been shown that the proper formation of the basal lamina by the Schwann cell is required prior to the production of myelin (Bunge, M.B. *et al*, 1990). If basal lamina formation is perturbed within this *in vitro* system, Schwann cells cannot

subsequently ensheath and myelinate axons within the system. Since myelin was present within cultures from all culture conditions, it can be assumed that appropriate basal lamina formation occurred regardless of infection status and temperature.

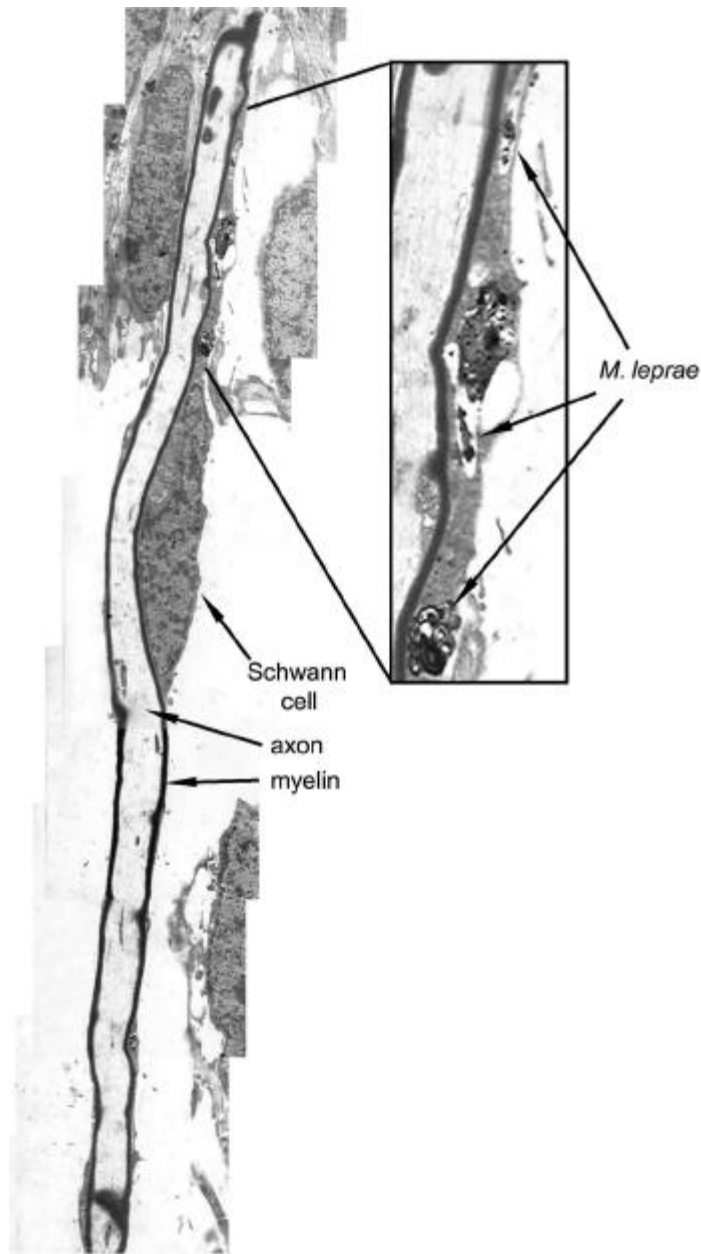


**Figure 4.4.** Transmission electron micrographs of horizontal sections of uninfected (A) and live *M. leprae*-infected (B) Schwann cell/neuron co-cultures maintained at 33<sup>0</sup> C. Purified neuronal cultures were seeded with uninfected or infected Schwann cells (SC) 12 d post-infection and then subsequently stimulated to produce myelin. Micrographs shown at approximately 5,000x (A) and 8,880x (B) final magnification.

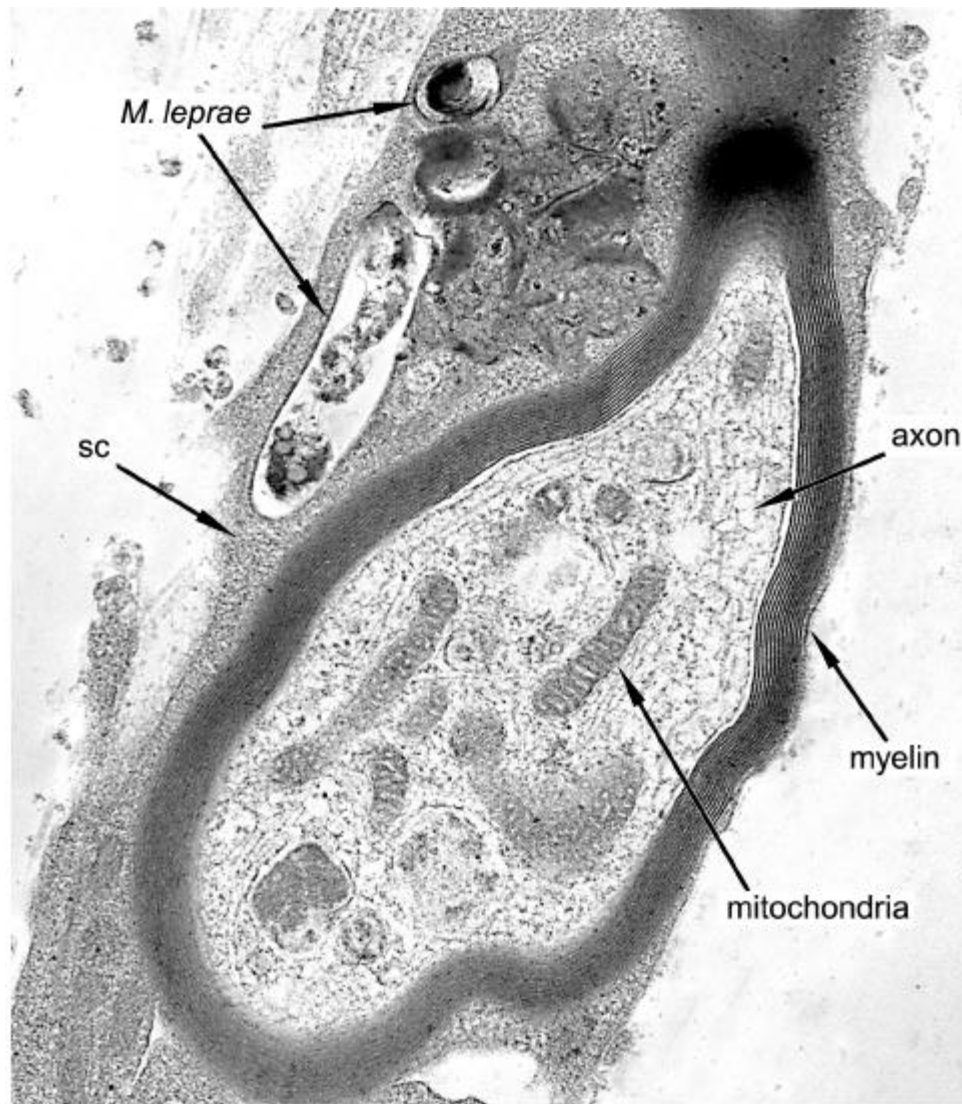




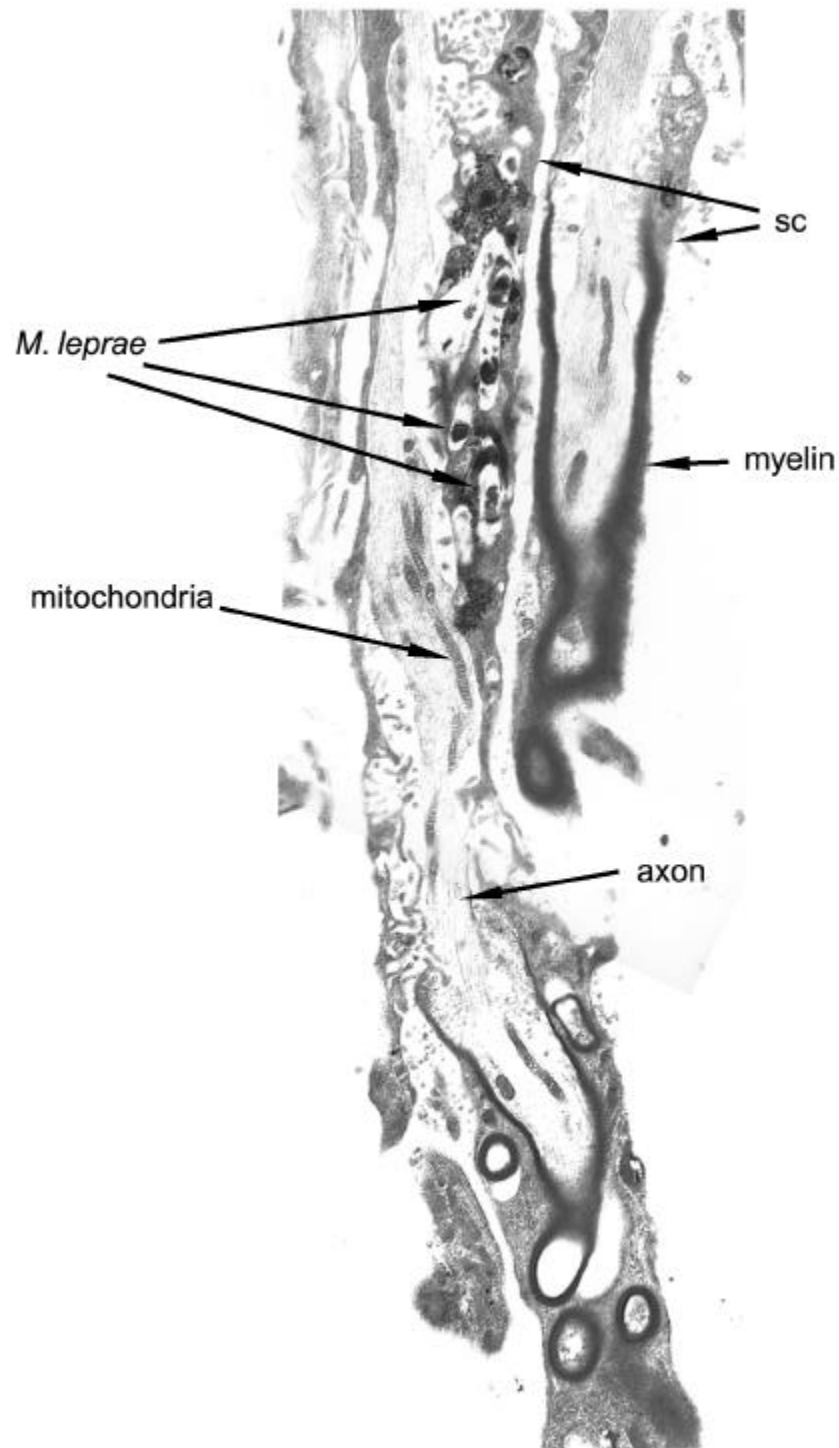
**Figure 4.5.** Transmission electron micrographs of cross sections of uninfected (A) and live *M. leprae*-infected (B) Schwann cell/neuron co-cultures maintained at 33<sup>0</sup> C. Purified neuronal cultures were seeded with uninfected or infected Schwann cells (SC) 12 d post-infection and then subsequently stimulated to produce myelin. Micrographs shown at approximately 9750x (A) and 4725x (B) final magnification.



**Fig.4.6.** A collage of transmission electron micrographs of a live *M. leprae*-infected Schwann cell/neuron co-culture. Infected Schwann cells (SC) maintained at 33<sup>0</sup> C were seeded onto purified neurons 12 d post-infection and then stimulated to produce myelin. The collage is shown at approximately 2,400x with an inset of 8,200x final magnification.

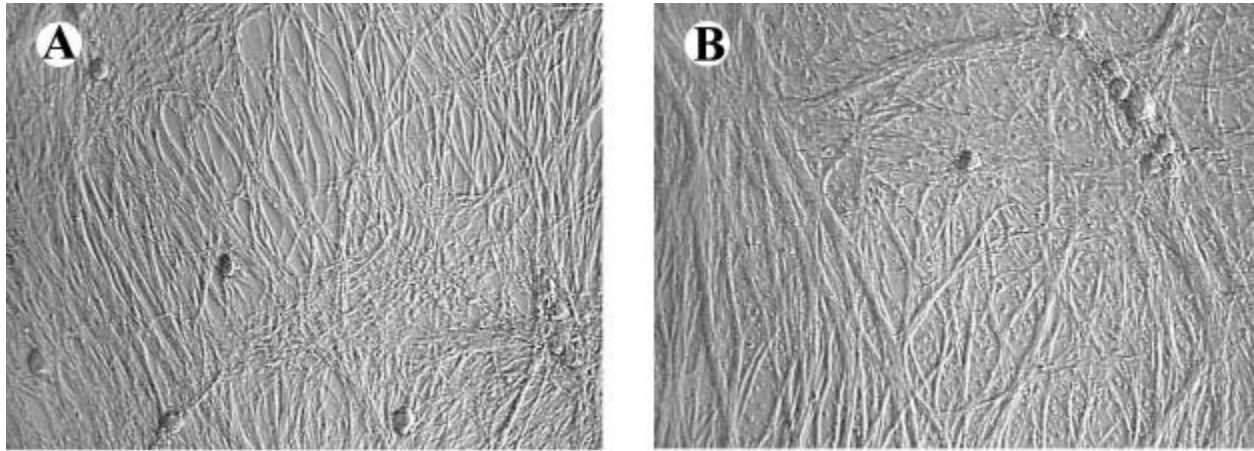


**Figure 4.7.** Transmission electron micrograph of a horizontal section of a live *M. leprae*-infected Schwann cell/neuron co-culture. Infected Schwann cells (SC) were seeded onto purified neurons 12 d post-infection and subsequently stimulated to produce myelin. Micrograph is shown at approximately 34,000x final magnification.



**Figure 4.8.** A collage of transmission electron micrographs of a live *M. leprae*-infected Schwann cell/neuron co-culture. Infected Schwann cells (SC) maintained at 37° C were seeded onto purified neurons 12 d post-infection and then stimulated to produce myelin. Collage shown at approximately 9,600x final magnification.

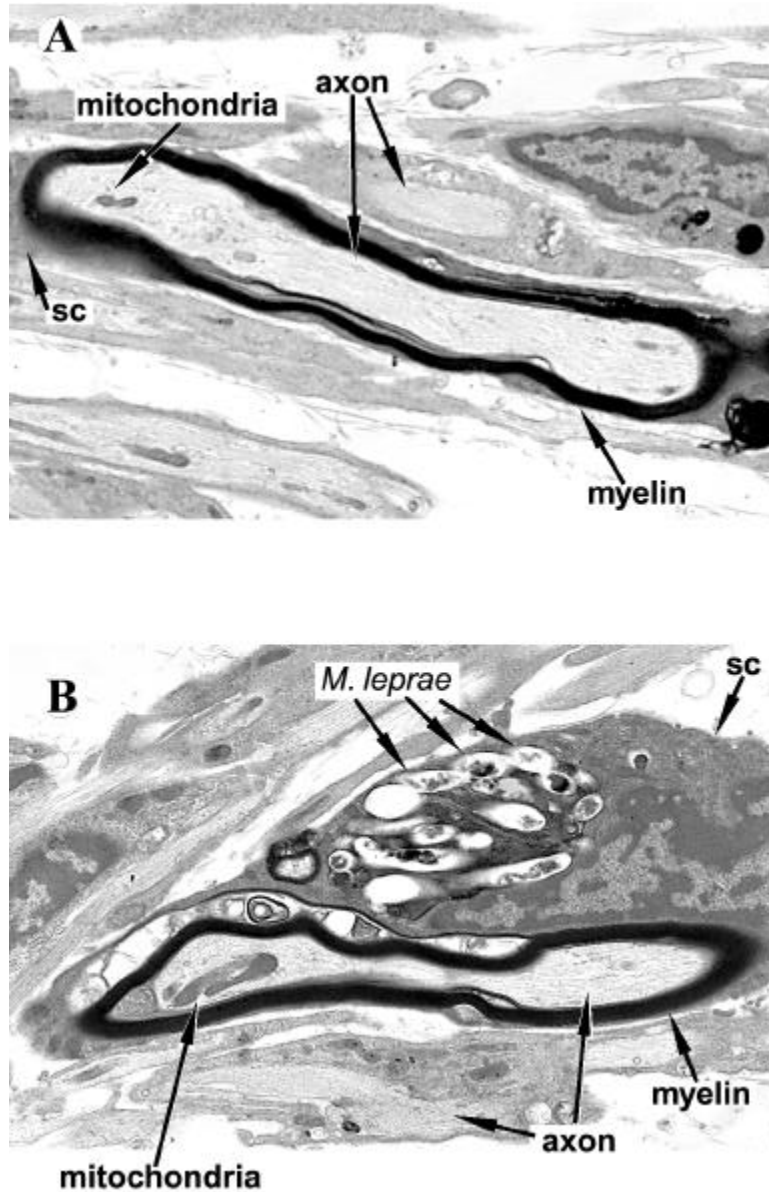




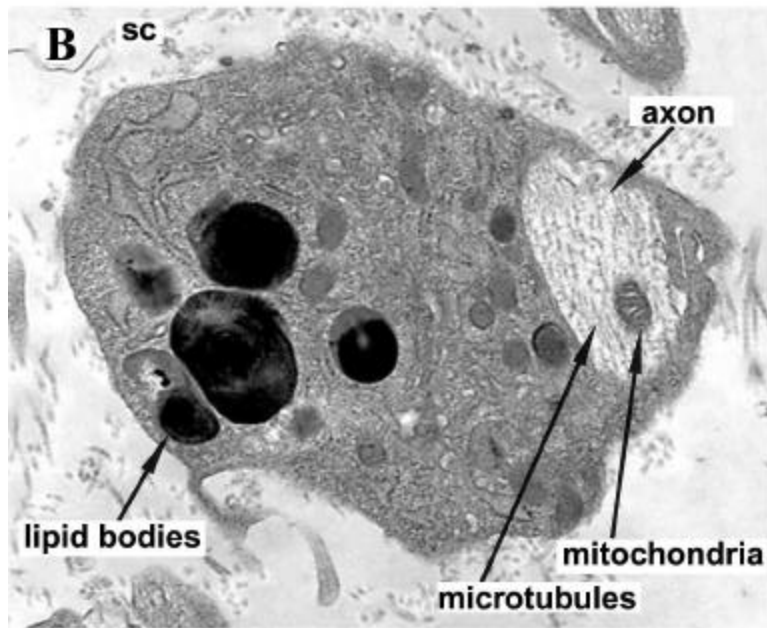
**Figure 4.9.** Phase contrast micrographs (10x original magnification) of uninfected (A) and infected (B) Schwann cell/neuron co-cultures at 33<sup>0</sup> C. Purified primary Schwann cells were seeded onto neurons, stimulated to myelinate, exposed to live *M. leprae* and maintained for 1 month post-infection.

In addition, myelin structure was similar under all conditions tested. No abnormalities in myelin structure such as “loosening” were observed. These data further support the observations of Jacobs *et al* (1987) which proposed that the “loosening” of myelin lamellae observed within *M. leprae*-infected biopsies more closely resembled damage done by immune bystander effects on myelin structure rather than that of myelin formation alteration demonstrated in other diseases.

Job (1971) reported that Schwann cells with increased bacillary load in the later stages of disease eventually became foamy, degenerated and contributed to the axonotrophy of the associated axon. Therefore since leprosy is slow to develop, time of infection, replication or number of the bacilli, which can number in the hundreds, within the host Schwann cell may influence neuropathy. The presence of multiple lymphokines and cellular infiltrates may also have an effect on this observation (Spierings, E. *et al*, 2001).

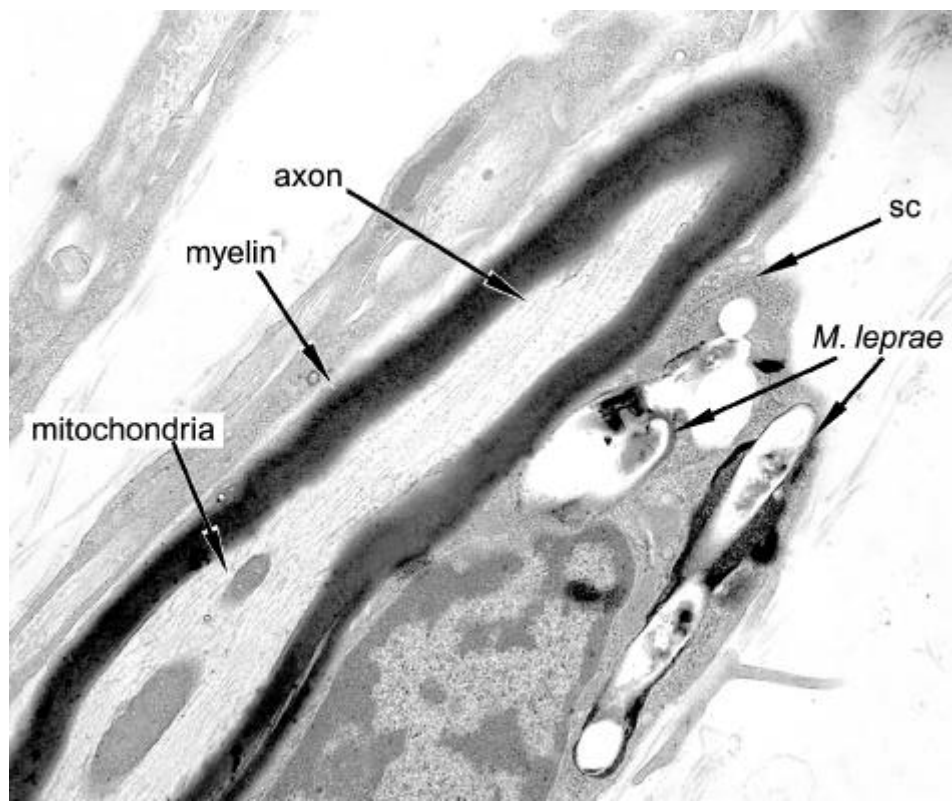


**Figure 4.10.** Transmission electron micrographs (4300x and 7400 x original magnification respectively) of uninfected (A) and metabolically active *M. leprae*-infected (B) Schwann cell/neuron co-cultures. Established co-cultures were maintained for 1 month post-infection at 33<sup>0</sup> C. Both micrographs are shown at approximately 8,000x final magnification.



**Figure 4.11.** Transmission electron micrograph of Schwann cell/neuron co-culture established and maintained at 33<sup>0</sup> C. The micrograph is shown at approximately 18,400x final magnification.

It has been hypothesized that the formation ‘foamy’ Schwann cells observed in lepromatous biopsies occurs as a result of higher bacillary loads which may contribute to the eventual destruction or dissolution of the Schwann cell (Job, C.K., 1971). The bacillary load within the Schwann cells in the present study (PI of 20) did not represent the extreme lepromatous stages of the disease which can demonstrate hundreds of bacilli per Schwann cell (Job, C.K. *et al*, 1971). Because the results of infection were analyzed one month post-infection, this study did not address the potential long term effects of infection since leprosy is a slow developing disease. Studies need to be conducted to simulate longer term infection with higher numbers of bacilli.



**Figure 4.12.** Transmission electron micrograph of a live *M. leprae*-infected Schwann cell/neuron co-culture. Established co-cultures were maintained for 1 month post-infection at 33<sup>0</sup> C. The micrograph is shown at approximately 14, 700x final magnification.

The microscopic methods used in this study allowed for intensive analysis of Schwann cell/*M. leprae* and Schwann cell/neuron interactions; however, the molecular level of interaction was not analyzed. Alteration of surface molecules or gene transcripts could have occurred as a result of infection; however if altered, the functional parameters dependent upon their expression such as alignment and association with neurons, proliferation along neurons and subsequent myelination were not altered at a level discernable by the methods of microscopy used within this study. Further analysis by immunomicroscopy or other molecular methods may reveal alterations that could not be shown by the methods used in this study.

The results of the present study indicate that in the early stages of infection, without the presence of immune factors produced from other cell types, Schwann cells retain functional capabilities. They appear to be capable of participating in the regenerative response and continue to maintain myelin sheath architecture. These data contradict earlier findings by Mukherjee *et al* (1981) which suggested that Schwann cells infected with *M. leprae* failed to associate with axons *in vitro* and do not subsequently produce myelin.

In Mukherjee *et al* experiments, Schwann cells were infected with *M. leprae* harvested from the nodules of untreated human lepromatous patients. This bacterial preparation was not tested to determine viability. In addition, infection was performed at 37<sup>0</sup> C, which is now known to rapidly reduce the viability of *M. leprae in vitro* (Truman, R.W. and Krahenbuhl, J.L., 2001) even within Schwann cells (**Chapter 2**). Therefore, the bacteria used for these experiments were most likely of low viability or dead during most of the functional assays.

Furthermore, infected cells in these cultures that were not fully associated with the axons were not conclusively identified as Schwann cells using classical immunostaining techniques such as the anti-S100 stain (Brookes, J.P., 1979). Nerve explants were used for their experiments, and although subjected to antimitotic treatment for fibroblast removal, it is virtually impossible to remove all fibroblast contamination from these cultures (Kleitman, N. *et al*, 1996). In addition, fibroblasts have been shown to internalize *M. leprae in vitro* and fibroblasts do not associate with axons *in vitro* (Mukherjee, R. *et al*, 198). Conversely, it is known that virtually 100% of Schwann cells associate with axons within 24 h when seeded onto neurons *in vitro* (personal communication with Dr. Ina Wanner at the Miami Project for the Cure of Paralysis, University of Miami Medical School, Miami, FL). Therefore, it is possible that infected cells in those experiments that failed to associate fully with axons were fibroblasts. Within the

experiment presented in this study using Schwann cells purified of fibroblasts and positively identified by anti-S100 immunomicroscopy, all Schwann cells regardless of infection status and temperature were shown to be functionally capable of associating and aligning with neurons *in vitro* in a manner comparable to controls.

In addition, Muhkerjee *et al* experiments reported that infected Schwann cells were rendered incapable of undergoing mitosis. Infected Schwann cells in the present study were capable of attaching and proliferating along axons. No apparent differences in proliferation were observed in infected cultures compared to controls. Therefore, our data contradict that of Muhkerjee, *et al* and indicate that *M. leprae* infection within the parameters investigated does not alter Schwann cell replication *in vitro*.

Therefore, the data obtained from the present study supports the hypothesis that *M. leprae* infection alone does not result in the neuropathology associated with leprosy. Therefore, the immune response to infection within the peripheral nervous system may bear more of the responsibility for the resulting neuropathology.

*M. leprae* may induce the Schwann cells to produce factors which recruit immune or inflammatory cells into the nerves. Schwann cells have been shown to produce neurotrophic factors as well as molecules with immunologic functions such as monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6), and interleukin-1 (IL-1) (Bergsteinsdottir, *et al*, 1991; Armati, P.L. and Pollard, J.D., 1996). Tumor necrosis factor-alpha (TNF $\alpha$ ) mRNA can also be produced by Schwann cells, though production of the molecule and IFN $\gamma$  has yet to be shown (Armati, P.L. and Pollard, J.D., 1996). It is likely that the infiltration of immune cells responding infection of the Schwann cell and accompanying fluids and the products that these cells produce ultimately leads to destruction of the nerve.

These experiments also highlight the critical need for a standardized *in vitro* Schwann cell infection model that retains *M. leprae* metabolic activity for the duration of the infection. By utilizing these *in vitro* conditions, the infection model can better mimic the conditions of live *M. leprae* infection *in vivo* and, thereby, contribute to a more thorough understanding of the early mechanisms of the nerve damage in leprosy.

## CHAPTER 5. CONCLUSIONS

The purpose of this research was to develop and characterize an improved *in vitro* model to study the effects of *Mycobacterium leprae* infection on Schwann cell and Schwann cell/neuron interactions that more closely mimics the *in vivo* environment. The improved model was then used to study the effects of infection on Schwann cell metabolic activity, morphology, gene expression and the ability of this cell to appropriately interact with cultured neurons. In addition, the model was used to determine the effects of the Schwann cell on the intracellular viability of *M. leprae*.

Since the viability of a pathogen may have a major impact on the outcome of infection, an improved model for studying *M. leprae*/Schwann cell interactions should include the use of highly viable *M. leprae* and maintenance of cultures at an incubation temperature that is not detrimental to its viability. *M. leprae* reside primarily in the cooler regions of the body in leprosy patients (30-34<sup>0</sup> C) and recent *in vitro* data suggest that the viability of *M. leprae* is preserved for longer periods of time at 33<sup>0</sup> C than at 37<sup>0</sup> C. Therefore, it was hypothesized that an improved *in vitro* model for studying the neuropathology of leprosy should include the use of highly viable *M. leprae* preparations for infection, rat Schwann cells and Schwann cell/neuron co-cultures and incubation of infected cultures at 33<sup>0</sup> C.

Results of these studies showed that 33<sup>0</sup> C incubation did not appear to have any adverse effects on Schwann cell growth, morphology, gene expression, or the ability of these cells to associate, proliferate and myelinate axons of cultured neurons. In addition, Schwann cells were able to maintain myelin sheaths in co-cultures at 33<sup>0</sup> C for months. This was also observed in co-cultures in which Schwann cells had been mitotically-expanded prior to seeding onto neurons. In addition, this model maintained the metabolic activity of *M. leprae* within the Schwann cells



for long periods of time. Therefore, it was concluded that an improved model for studying the effects of *M. leprae* infection on Schwann cells had been developed.

When this model was applied to study the effects of *M. leprae* infection on Schwann cells, the results demonstrated that Schwann cells exhibited a differential response when maintained at 33<sup>0</sup> C compared to 37<sup>0</sup> C. *M. leprae*-infected Schwann cells exhibited an altered monolayer morphology and gene expression, primarily in those genes encoding Schwann cell adhesion molecules when they were held at 33<sup>0</sup> C. These results suggested that altered expression of these molecules resulted in altered Schwann cell/Schwann cell interactions. In addition these results suggested that infection may alter the Schwann cell's ability to appropriately interact with axons, and thereby, affect the ability of the Schwann cell to participate in the nerve regenerative process *in vivo*.

These experiments indicated that *M. leprae* metabolic activity was affected by the change in temperature to 33<sup>0</sup> C, but did not conclusively determine if the Schwann cell was altered on a molecular level by the temperature change. Functional analyses and gross morphological observations did not indicate that alteration within the Schwann cell was due to the lower incubation temperature. Therefore within the parameters of this study, the alteration of Schwann cell response to infection directly correlates with *M. leprae* metabolic activity; however, the combination of infection along with temperature affects on the Schwann cannot be ruled out as a contributing factor.

Altered gene expression as a result of the metabolically active *M. leprae*-infection of the Schwann cell was observed. Glial fibrillary acidic protein (GFAP) and neural-cadherin (N-cadherin) gene transcripts were significantly decreased post-infection with metabolically active *M. leprae*, and neural cell adhesion molecule (NCAM) gene transcripts were shown to have

significantly increased post-infection with metabolically active *M. leprae*. These alterations of important cell surface molecules critical to the function of the Schwann cell and interaction with neurons could potentially alter the functional capabilities of the Schwann cell in regard to nerve regeneration as well as myelin production and maintenance.

Transforming growth factor-beta 1 (TGF $\beta$ 1) transcript levels were altered; however, only within cells containing dead *M. leprae* at both 33<sup>0</sup> C. The significant lower levels of transcripts may be indicative of a suppression of the Schwann cell response the intracellular presence of the bacteria. TGF $\beta$ 1 is an important cell mediator to trauma (Einheber, S. 1995).

Irreversible nerve damage has been shown to continue to develop during and even after antimicrobial therapy (Gillis, T.P and Hasting, R.C., 1992; Rambukkana, A., 2000). It is estimated that therapeutic intervention has prevented only one-third of infected individuals from suffering further nerve damage (World Health Organization, 1998). The potential suppression of TGF $\beta$ 1 expression by the Schwann cell may indicate an impairment of the Schwann cell to appropriately respond to the intracellular dead *M. leprae* and therefore may contribute to the subsequent neuropathy observed even after effective multi-drug therapy.

Even though data from the above experiments suggested that *M. leprae* has an apparent, detrimental effect on Schwann cell/Schwann cell interactions, *M. leprae*-infected Schwann cells were able to attach, align with axons in culture, proliferate along these axons and subsequently differentiate into the myelinating phenotype similar to those observed in control cultures. Therefore, these data suggest that infected Schwann cells are capable of participating in the nerve regenerative processes such as attaching to 'naked' axons, aligning and proliferating along axons and differentiating into the myelinating phenotypes to re-myelinate injured nerve fibers.

Established myelinated Schwann cell co-cultures, subsequently infected with *M. leprae*, did not appear to sustain any apparent damage to myelin sheaths or other cellular organelles after one month of infection. These results suggested that *M. leprae* infection at this MOI alone does not appear to alter the Schwann cell's ability to maintain its myelin sheath architecture. Therefore, the alteration of myelin sheaths observed in early leprosy nerve fibers does not appear to be a direct result of *M. leprae* infection.

Previous studies of lepromatous patient biopsies have demonstrated foamy or degenerating Schwann cells containing hundreds of bacilli per cell (Job, C.K., 1971). The multiplicity of infection (MOI) used within these experiments, however, resulted in an average phagocytic index (PI) of only 20 *M. leprae* per Schwann cell. This PI is not representative of the bacillary loads observed in lepromatous biopsies nor that seen within tuberculoid biopsies (Adams, L.B. and Krahenbuhl, J.L., 1996). Within an *M. leprae* lepromatous infection, the lack of a limiting immune response allows for the extreme proliferation of *M. leprae* within the nerve (Adams, L.B. and Krahenbuhl, J.L., 1996). Neuropathy is thought to be due to heavy bacillary loads or bacillary multiplication within Schwann cells. Therefore within the parameters of this study, it can be stated that metabolically active *M. leprae* infection of the Schwann cell at this MOI for at least one month post-infection does not result in apparent neuropathy. Further analyses of Schwann cells infected for longer periods of time with greater bacillary loads may demonstrate neuropathy not seen within these experiments.

In summary, these results not only support the use of the 33<sup>0</sup> C infection model, but provide evidence that the histopathology observed within human leprosy lesions may not be due to the direct effects of *M. leprae* within the Schwann cell. It appears that *M. leprae* infection

alone is fairly innocuous to the Schwann cell and that the Schwann cell is incapable of killing intracellular *M. leprae*, in the absence of an immune factors within the nerve.

Evidence from other demyelinating neuropathies frequently implicates the immune response as a mechanism of pathology within the nerve. Cytokines such as TNF $\alpha$  and IFN $\gamma$  have been shown to have direct effects on myelin protein gene expression (Schneider-Schaulies, J. *et al*, 1991). Macrophages have been shown to have a support role in the removal of myelin from damaged nerves (Ide, C., 1996).

Cytokines, immune cells, and other immune factors within leprosy lesions have been shown to have distinctive roles within leprosy lesions. However, prior to the evidence presented within this dissertation, no comprehensive studies had been performed to investigate the role of the direct effects of viable *M. leprae* upon the Schwann cell and Schwann cell function. Because the data from this study imply that intracellular *M. leprae* has no apparent direct effect on the Schwann cell functional capabilities in the absence of immune factors, research emphasis should be placed on defining the role of immune bystander effects on infection within this model. In addition, the effects of *M. leprae* infection on Schwann cell cytokine expression such as interleukin-1, interleukin-6, TNF $\alpha$ , IFN $\gamma$  and monocyte chemoattractant protein-1 should be investigated; because it is likely that the presence of intracellular *M. leprae* stimulates cytokine production by Schwann cells which may lead to recruitment of immune cells into the nerve (Armati, P.J. and Pollard, J.D.1996). Although within a lepromatous response, which typically lacks an aggressive Th1 immune response, neuropathy may still be the result of higher bacillary load, multiplication of bacilli within the Schwann cell and prolonged infection (Adams, L.B. and Krahenbuhl, J.L., 1996).

Current leprosy control regimens consist of multi-drug treatment of identified infections. In addition research is currently being conducted to develop a leprosy vaccine which could be used to prevent infection and treat existing infections by stimulating a Th1 cellular and cytokine immune response to *M. leprae*. However, if neuropathology associated with leprosy results from an aggressive immune response within the nerve and not as a direct consequence of Schwann cell destruction by *M. leprae*, as indicated by the findings of this study, it is possible that induction of these responses may stimulate further neuropathy rather than prevent it.

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## VITA

Deanna Alisa Hagge was born in Lawrence, Kansas to Stephen Charles Hagge and Margaret Dean Hagge. As her father pursued his career in the Navy and then transitioned to civilian life as an electrical engineer, the family moved many times and finally came to settle near Baton Rouge, Louisiana when Deanna was 13, around which time the family had also begun to homeschool. By the time Deanna graduated, she had four younger brothers: Brian, Kent, Jeremy and Stefan.

After graduation, she began studies at Louisiana State University with an LSU Honor Scholarship and Chancellor's student aid position as well as the Robert C. Byrd Scholarship from the Louisiana State Board of Elementary and Secondary Education. As an undergraduate, she taught freshman chemistry laboratory for three semesters. In 1996, she graduated *magna cum laude* with a degree in microbiology and a minor in chemistry. The following fall, she began to pursue her Ph.D. in microbiology with a minor in biochemistry. She has taught microbiology lab for three semesters and plans to graduate this year.

Deanna currently lives in Zachary, Louisiana and is a member of Community Christian Center where she is involved in the worship and children's ministry. She looks forward to living life fully in Jesus (Philippians 3:12-14), knowing that He guides (Isaiah 30:21), provides (Matthew 6:25-33) and has a purpose for her life (Ephesians 2:22; 2 Corinthians 1:3-4; Matthew 28:19-20). She plans to follow and love Him faithfully to the end of her days and beyond.